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**PRENATAL DEXAMETHASONE EXPOSURE:
GLUCOCORTICOID PROGRAMMING OF THE BRAIN**

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Declaration

I declare that this thesis is a presentation of my original research work.

Wherever contributions of others are involved, every effort is made to indicate this clearly.

The data presented in this thesis has not been submitted for any other degree.

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List of abbreviations

-RT	No reverse transcriptase sample
11 β -HSD2	11 β -hydroxysteroid dehydrogenase type II
ACTH	Adrenocorticotrophic hormone
AD	Autistic disorder
ADHD	Attention deficit hyperactivity disorder
Arc	Activity-regulated cytoskeletal-associated protein
AS	Angelman syndrome
ASD	Autism spectrum disorder
Avp	Arginine vasopressin
Bdnf	Brain-derived neurotrophic factor
BRF	Biomedical Research Facility
CAH	Congenital adrenal hyperplasia
CD	Compound discrimination
Cdkn1c	Cyclin dependent kinase inhibitor 1c
cDNA	Complimentary DNA
CDS	Coding DNA sequence
CGAT	Computational Genomics Analysis and Training
Cnr1	Cannabinoid receptor-1
CORT	Corticosterone
CpG	Cytosine-phosphate-Guanine
Crhr2	Corticotrophin-releasing hormone receptor 2
DEPC	Diethyl pryrocarbonate
Dex	Dexamethasone
Dio3	Iodothyronine deiodinase 3
Dll1	Delta-like homolog 1

DMRs	Differentially methylated regions
DNA	Deoxyribonucleic acid
Dnmt	DNA methyltransferases
ED	Extra-dimensional discrimination
Egr1	Early growth response 1
ELISA	Enzyme-linked immunosorbent assay
G6pd	Glucose-6-phosphate dehydrogenase deficiency
Gad67	Glutamic acid decarboxylase 67
GLMs	Generalised linear models
Gnas	Guanine nucleotide binding protein a-stimulating
GR	Glucocorticoid receptor
Grb10	Growth receptor bound protein 10
Gtl2	Gene-trap locus 2
Hnf4 α	Hepatic nuclear factor 4 α
HPA	Hypothalamic-pituitary-adrenal
Hprt	Hypoxanthine-guanine phosphoribosyl transferase
ID	Intra-dimensional discrimination
ID/ED	Intra-dimensional/Extra-dimensional
IEGs	Immediate early genes
IG-DMR	Intergenic germline-derived DMR
Igf2	Insulin-like growth factor type 2
Igf2r	Igf2 receptor
INS	Insulin
ITI	Inter-trial interval
L1	Long interspersed element-1
lncRNAs	Long non-coding RNAs

LTP	Long-term potentiating
LTR	Long terminal repeat
MBDs	Methyl-CpG binding-containing proteins
Mecp2	Methyl CpG-binding protein 2
MEG3	Maternally expressed gene 3
MEST	Mesoderm specific transcript
miRNAs	MicroRNAs
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
ncRNAs	Non-coding RNAs
NGFI-A	Nerve growth factor inducible gene-A
NMDA	N-methyl-D-aspartate
ORFs	Open reading frames
PCR	Polymerase chain reaction
PGCs	Primordial germ cells
Ppar	Proliferator-activated receptor
PSD	Postsynaptic density
PVN	Paraventricular nucleus
PWS	Prader–Willi syndrome
qPCR	Quantitative real-time PCR
Rev	Reversal
RIN	RNA Integrity Number
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RNAPII	RNA polymerase II
rpm	Revolutions per minute

RT	Reverse transcription
RT°	Room temperature
S.D.	Standard deviation
S.E.M.	Standard error of mean
SD	Simple discrimination
SH2	SRC homology domain 2
siRNAs	Small interfering RNAs
Snrpn	Small nuclear ribonucleoprotein polypeptide N
snoRNA	Small nucleolar RNA
SRS	Silver-Russell syndrome
Tbp	TATA box binding protein
TEs	Transposable elements
Th	Tyrosine hydroxylase
Ube3a	Ubiquitin-protein ligase E3A
UPL	Universal Probe Library™
UTR	Untranslated region

Abstract

Human epidemiological studies have provided compelling evidence that prenatal environmental events are associated with significantly increased risks of developing neuropsychiatric disorders in adulthood. This phenomenon has been studied and known as ‘fetal programming of adult diseases’. According to the theory, environmental insults occurring at critical periods of pregnancy can permanently alter the structure and physiology of the developing organs, and may lead to adverse functional consequences that manifest later in life. It has been suggested that increased maternal glucocorticoids may be one common mechanism through which various environmental insults can affect on the developing fetus. Therefore, in this thesis, I studied the long-term prenatal glucocorticoid overexposure effects on the functioning of the brain and investigated possible molecular mechanisms underlying the programmed effects.

By using a rat model of prenatal dexamethasone (100µg/kg per day) administered to pregnant dams during the last week of gestation, I found that prenatal dexamethasone overexposure significantly reduced birth weight in both male and female offspring. I also assessed the consequences of prenatal dexamethasone treatment on stress response and cognition in the adult male offspring by using a number of physiological and behavioural measures. Overall I found no evidence of prenatal dexamethasone treatment effect on the hypothalamic-pituitary-adrenal (HPA) axis activity and behavioural responsiveness to an acute stress in adult male offspring; however, prenatal dexamethasone exposure affected hippocampus and prefrontal cortex related cognition in the adult offspring, including contextual fear conditioning and behavioural flexibility on water maze tasks. I further explored possible molecular mechanisms that underlie the behavioural phenotypes of the prenatal dexamethasone rat model, and found altered brain gene expression with possible implications in synaptic plasticity and retrotransposon activities.

Collectively, my current study suggests that prenatal dexamethasone exposure induces long-term programming effects on adult behaviour, associated with altered gene expression profile of the brain in the rat offspring. These results provide further evidence that prenatal glucocorticoid exposure may affect the development of the brain and its influence may endure into adulthood.

Lay summary

Environmental adversities during pregnancy are believed to affect the baby's brain development and later mental health. Possible environmental insults range from everyday stressful life events to the more severe and rare natural disasters. The prenatal effects on the brain may not be apparent at birth, but develop as the child grows. There is evidence that children of mothers who experienced higher levels of anxiety during pregnancy are likely to have more emotional and behavioural problems, and there is a bigger chance for them to develop mental problems when they become adults. How these environmental events affect are not yet clear, but effects of maternal stress hormones on the developing fetal brain may be one mechanism through which a range of environmental stressors affect the development and function of the brain. In clinic, hormone-based treatments are used in several clinical conditions during pregnancy. While this therapy has undoubtedly improved infant health, the long-term safety of prescribing hormone in pregnant women is lacking.

In this project I used a rat model to find out the possible long-term impact of prenatal treatment of stress hormones on the function of the brain. My results suggest that overexposure to stress hormones during pregnancy had an enduring effect on the brain, producing long-term alterations in behaviour and brain gene expression in the adult male rat offspring.

Chapter 1 Introduction

1.1 Fetal programming of adult diseases

Environmental factors can act on the genetic constitution of an organism to drive polyphenism, a phenomenon that has been studied by biologists since the 19th century. Striking examples include developmental and reproductive transitions in plants and animals that are brought about by natural selection pressures, such as predators, temperature changes, resource availability, chemical pollutants and parasitism. For example, it has been reported that developmental temperature induces physiological traits that help an organism to tolerate extreme climates (Sinclair et al., 2003). Nutritional availability of the larvae during the first three days of life determines the functional differentiation of the honeybee (Weaver, 1955). Similarly, a number of factors such as sex hormones, temperature and parasitism are known to drive sexual dimorphism in species including invertebrates, fishes, amphibians, birds and mammals (Bergerar.J, 1972).

These studies provide important evidence that environmental influence during a specific time of development can ‘program’ the end result of phenotype. However, the term ‘programming’ was not officially acknowledged until the late 20th century, when Barker first introduced the idea of ‘fetal programming of adult diseases’ by showing a link between low birth weight and increased deaths from ischaemic heart disease in adulthood (Barker, 1990). The concept of ‘fetal programming’ is used to describe the fact that early life environmental factors can affect fetal development and predict adulthood diseases. According to the theory, environmental insults during sensitive periods of development can permanently affect the structure and physiology of the developing organs that are concurrently vulnerable, and thus induce lifelong consequences on health (Seckl and Holmes, 2007). This kind of developmental plasticity has been proposed to help the unborn animal adapt its body systems for better survival in a potentially hazardous postnatal environment. However, if a mismatch occurs between the

intrauterine environment and the later adulthood environment, such an effort is likely to cause maladaptation and result in a higher risk for diseases (Gluckman et al., 2007).

1.1.1.1 Programming of neuropsychiatric disorders

Multiple lines of evidence suggest that prenatal environmental factors have programming effects on the developing fetus, and that prenatally exposed individuals are at increased risks of developing a number of adult diseases in later life. While earlier studies focused preferentially on the metabolic and cardiovascular outcomes of intrauterine adversity, more recent studies provided evidence that an adverse *in utero* environment is also predictive of adulthood neuropsychiatric disorders, including autism spectrum disorders (ASD), schizophrenia and attentional deficit hyperactivity disorder (ADHD) (Kinney et al., 2008b, Kinney, 2000, Linnet et al., 2003). These diseases are also called ‘neurodevelopmental diseases’, highlighting the fact that altered neural development is involved in the aetiology of the disease, and that neuropathological changes exist long before the onset of clinical symptoms.

Here I will discuss human epidemiological studies from different parts of the world and ethnicities, providing important evidence that a number of prenatal environmental factors are associated with increased susceptibility to neuropsychiatric disorders in adulthood.

1.1.1.1.1 Maternal stressful life events

In 1976, Ward reviewed data from maternal records of 59 pairs of autistic children and healthy children. In his report, 32% of mothers of autistic children (19 out of 59) reported having had family discord during the pregnancies, whereas only 2 out of 59 matched control mothers reported having experienced discord during their pregnancies ($P < 0.05$) (Ward, 1990). In a similar study, researchers surveyed 188 mothers of autistic children and a control group of 202

mothers of typical developing children on incidence of stressful life events experienced during their pregnancies. The reported presence of stressors during pregnancies was significantly higher in the autistic group (32.4 per 100 responses) compared to the control group (25.9 events per 100 responses; $P=0.0007$) (Beversdorf et al., 2005).

In another retrospective study, researchers studied the Finnish population register between 1925 and 1957, and identified an index group of 167 subjects whose fathers had died before their children's births and a control group of 168 subjects whose fathers had died during the first year of their children's lives. It has been found that the number of diagnosed schizophrenics was six times higher in the index than in the control group (Huttunen and Niskanen, 1978). In another study, Myhrman and colleagues studied the correlation between wanted versus unwanted pregnancies and the prevalence of later schizophrenia. Data were collected prospectively on 11,017 subjects from the 1966 Northern Finland birth cohort. The risk of later schizophrenia was significantly greater (relative risk of 2.4) in subjects born of unwanted pregnancies compared to those born of wanted pregnancies (Myhrman et al., 1996).

Two other studies prospectively investigated the association between maternal stress and ADHD risks. Van den Bergh and Marcoen (2004) conducted a survey by handing out the State Trait Anxiety Inventory to 71 mothers during their pregnancy. Their offspring were followed up at age 8 or 9 and ADHD symptoms were rated by the child's mother, school teacher and an external observer. Statistical analysis revealed that maternal anxiety during pregnancy accounted for 22%, 15% and 9% of the variance in cross-situational ADHD symptoms, externalizing problems and self-reported anxiety, respectively (Van den Bergh and Marcoen, 2004). In another prospective longitudinal study, researchers followed 290 participants from their first antenatal care visit between 1992 and 1994 in Uppsala County, Sweden. Maternal stress was recorded at different time points during pregnancy and their children were rated at 7 years old by the mother and school teacher on 18 symptoms related to ADHD according to

the DSM-IV criteria. The results indicated that ADHD diagnosis was significantly related to prenatal exposure to stress (Rodriguez and Bohlin, 2005).

1.1.1.2 Natural disasters and war

In one study, Kinney and colleagues studied the prevalence of autism in children that had been prenatally exposed to hurricanes and tropical storms that struck Louisiana between 1980 and 1996. They found that prenatal exposure to storms was significantly associated with increased risks of developing autism, and the prevalence of autism increased in a dose-response fashion with the severity of storm exposure (Kinney et al., 2008a).

In another study, researchers investigated the prevalence of schizophrenia in birth cohorts that had been prenatally exposed to the May 1940 military German invasion of the Netherlands in World War II. Individuals whose mothers were pregnant at the time of the invasion were identified as the index group, and individuals born in the adjacent two years before or after were identified as controls. These cohorts were followed up through the National Psychiatric Case Register over the period 1970-1994, and the index group was found to have a significant increased risk of lifetime diagnosis of schizophrenia (relative risk 1.15) (van Os and Selten, 1998).

1.1.1.3 Malnutrition

Studies also suggest that maternal famine during pregnancy is a risk factor for later schizophrenia. The 1944-1945 Dutch Hunger Winter was a time of severe food deprivation for the Dutch population following a Nazi blockade in western Holland. By reviewing data on population of famine exposure and schizophrenia hospitalization at age 24 and above in the exposed and unexposed birth cohorts from the national psychiatric registry, researchers reported a significantly increased risk of schizophrenia in birth cohort that had been exposed

to the famine during early gestation in both men and women (relative risk 2.0) (Susser et al., 1996). Similar result was obtained by another group, who studied the effects of the 1959-1961 famine in the Wuhu region of Anhui, China. The prevalence of schizophrenia was significantly increased in cohorts that had been prenatally exposed to the famine, with a mortality-adjusted relative risk of 2.30 for those born in 1960 and 1.93 for those born in 1961 (St Clair et al., 2005).

In summary, converging lines of research suggest that the risk of developing adulthood neuropsychiatric disorders is significantly increased following prenatal exposure to various environmental insults, including maternal stressful life events, natural disasters, war and maternal famine.

1.1.2 Prenatal stress and postnatal abnormalities

Apart from fully diagnosed neuropsychological diseases, there is evidence that prenatal stress can lead to persistent neuropsychological abnormalities that resemble central symptoms of neuropsychiatric disorders in humans (Dawson et al., 2000). However, insights from human studies have been limited, partly due to the high degree of individual variability and the confluence of uncontrolled environmental factors in human studies. Animal studies, on the contrary, can establish a direct correlation between prenatal stress exposure and long-term postnatal outcomes, when all the other confounding variables are tightly controlled under experimental conditions. By directly inducing standardized stress at specific times of gestation to the pregnant dams on laboratory animals, these studies have a potential to document a diversity of short-term and long-term physiological, behavioural and neurobiological outcomes in the offspring.

1.1.2.1 Physiological outcomes

Data from studies using different types of gestational stress in rodents and non-human primates indicated that prenatal stress reprograms the hypothalamic pituitary-adrenal (HPA) axis in the offspring. HPA axis is the neuroendocrine system that regulates the physiological reaction of the body to stress. Despite some inconsistency, the majority of studies report increased sensitivity of the HPA axis in the offspring of stressed dams, including increased basal secretion and/or enhanced stress-induced secretion of stress hormones (detailed in Chapter 3).

1.1.2.2 Behavioural outcomes

A large body of evidence suggest prenatal stress induces long-term behavioural changes in the offspring (Weinstock, 2008). Animals exposed to stress *in utero* exhibited increased expression of fear and anxiety when challenged with stressful stimuli (Vallee et al., 1997, Takahashi et al., 1992, Welberg et al., 2001, Wakshlak and Weinstock, 1990, Clarke et al., 1996). Offspring of stressed dams were also found to have cognitive deficits, including impairments in spatial reference memory (Lemaire et al., 2000, Son et al., 2006, Yang et al., 2006, Brabham et al., 2000, Markham et al., 2010), working memory (Markham et al., 2010, Gue et al., 2004), context- and cue-dependent fear conditioning and extinction (Markham et al., 2010, Green et al., 2011b, Wilson et al., 2013). In addition, offspring of stressed dams were also found to have reduced attention span, altered motor responses, amplified amphetamine self-administration and increased locomotor responses to novelty (Son et al., 2007, Deminiere et al., 1992, Diaz et al., 1997, Koenig et al., 2005, Schneider et al., 1999).

1.1.2.3 Neurobiological outcomes

Fear and stress-related pathological changes in prenatally exposed animals have been associated with altered functioning of the amygdala (Welberg et al., 2001, Weinstock, 1997),

decreased central glucocorticoid receptors expression (Welberg et al., 2001, Henry et al., 1994, Maccari et al., 1995, Brabham et al., 2000, Levitt et al., 1996) and altered brain neurotransmitter systems, including the serotonergic (Huang et al., 2012), dopaminergic (Son et al., 2007), catecholaminergic (Takahashi et al., 1992, Green et al., 2011b), cholinergic (Emgard et al., 2007), endogenous opioid and GABAergic systems (Weinstock, 1997).

There is also evidence that prenatal stress leads to long-lasting changes in hippocampal structure and function, associated with impaired spatial learning and memory in rat offspring. The changes include reduced hippocampal weight (Szuran et al., 1994, Mandyam et al., 2008), reduced hippocampal neurogenesis (Lemaire et al., 2000) and altered hippocampal synaptic plasticity as examined by electrophysiological and biochemical analysis (Yang et al., 2006, Son et al., 2006).

Moreover, there is evidence that prenatal exposure to stress during late gestation causes male-specific deficits in prefrontal cortex maturation in adolescent rats, associated with impaired cognitive behaviours related to prefrontal cortex (Markham et al., 2013). A microarray study of the same animal model indicated aberrant expression of genes involved in synaptic plasticity in the prefrontal cortex of adult offspring that have been prenatally exposed to stress (Kinnunen et al., 2003).

Taken together, the above evidence suggests that intrauterine experience can induce a permanent impact on brain structure and function, and affected individuals are at increased risks of developing lifetime psychopathological abnormalities.

1.1.3 Importance of timing

The importance of the timing in fetal programming has been suggested by several epidemiological studies listed in 1.1.1. These studies suggest that an insult may affect if it occurs during certain periods of gestation, and may have little or no effect if it occurs during other periods, although different types of insults (maternal anxiety versus maternal famine) may act differently if they occur at similar stages of pregnancy.

In the study by Kinney and colleagues, the increased risk for autism was 3.83 times greater in children who had been exposed to storms during gestation months 5-6 or 9-10 (equals to weeks 20-24 or 36-40) compared to children who had been exposed to the same storms, in the same place, but during other months of gestation ($p < 0.001$) (Kinney et al., 2008a). In the study by Beversdorf and colleagues, a higher incidence of prenatal stressors was found at 21-32 weeks of gestation, with a peak at 25-28 weeks, in the autism cohort (Beversdorf et al., 2005). In the Huttunen and Niskanen study for schizophrenia risk, all index subjects were, at the time when their fathers died, in weeks 9-20 or 33-40 of gestation (Huttunen and Niskanen, 1978). In the May 1940 German invasion of the Netherlands study, incidence of schizophrenia was found higher in men and women exposed during the first trimester and in men only exposed during the second trimester (van Os and Selten, 1998). In the Dutch hunger study, maternal famine during the first trimester was linked to increased risk for schizophrenia (Susser et al., 1996), whereas middle to late gestational famine was associated with major affective disorders (Brown et al., 2000). In addition, in the Van den Bergh and Marcoen study, maternal anxiety during gestational weeks 12-22, but not other gestational weeks, was linked to ADHD risk (Van den Bergh and Marcoen, 2004).

Overall the above evidence suggests that stressors during gestational weeks near the middle of gestation or before birth are linked to the development of ASD (Kinney et al., 2008a,

Beversdorf et al., 2005), exposures during early (van Os and Selten, 1998, Susser et al., 1996), middle (van Os and Selten, 1998, Huttunen and Niskanen, 1978) and late gestation (Huttunen and Niskanen, 1978) are linked to schizophrenia, whilst second trimester exposure only is associated with the development of ADHD (Van den Bergh and Marcoen, 2004). These programming windows may represent vulnerable stages and different regions of brain development, and perturbations during these sensitive periods may disrupt the normal series of developmental events and lead to adverse functional consequences that manifest later in life.

1.2 Glucocorticoid programming

There are many hypotheses regarding how the programming effects of prenatal stress communicate to the offspring. Theories that have been proposed include a direct action on the fetus through mediating factors, or an indirect action by affecting placenta function or postnatal maternal behaviour. Among these, fetal exposure to excessive maternal glucocorticoids represents one of the most plausible links between prenatal stress and increased susceptibility to later on diseases (Seckl and Meaney, 2004).

According to the glucocorticoid programming theory, stress experienced by the pregnant mother activates the HPA axis, leading to increased glucocorticoids levels in the maternal circulation that may cross the fetoplacental barrier and act on the developing fetus. It has been well recognized that other physiological steroids, such as sex hormones, can have programming effects on the developing fetus, with an influence that persists throughout life (Gustafsson et al., 1983). Thus, glucocorticoids may act in a similar way as ‘programming factors’ and have the potential to communicate various environmental stressors to the fetus.

The rationale of glucocorticoid programming can be found a) from the link between low birth weight and increased risks of adult diseases and b) from a number of animal studies, which provide direct evidence as to the effects of maternal glucocorticoids administration on the offspring, as detailed below.

1.2.1 Evidence supporting the role of glucocorticoids in programming

The strongest evidence of glucocorticoid programming comes from the link between low birth weight and increased risks of adulthood diseases. The association between low birth weight and subsequent development of cardiovascular and metabolic disorders in adulthood was first documented by Barker in 1990 (Barker, 1990), and since then found evidence in a number of other epidemiological studies (Hales et al., 1991, Law et al., 2002, Leon et al., 1996, Stein et al., 1996). In addition, recent evidence indicates that low birth weight is also predictive of an increased risk for neuropsychiatric disorders in adult life, including stress-related disorders (Lundgren et al., 2001, Nilsson et al., 2004), autism (Pinto-Martin et al., 2011), schizophrenia (Abel et al., 2010, Freedman et al., 2013) and ADHD (Breslau et al., 1996). The association between low birth weight and adulthood diseases is largely independent of other lifestyle risk factors, and are continuous within the normal birth weight range (Barker, 1993, Abel et al., 2010).

This evidence complements the epidemiological studies discussed in 1.1.1, suggesting that an aversive fetal environment may lead to both intrauterine growth restriction and increased susceptibility to adulthood diseases. Low birth weight, if considered another phenotype programmed by prenatal adversity, supports the role of glucocorticoids in fetal programming. In humans, placental 11β -hydroxysteroid dehydrogenase type II (11β -HSD2) catalyses the conversion of active glucocorticoids into their inert counterparts and it has been suggested that inter-individual variations in 11β -HSD2 activities negatively correlates with the amount of glucocorticoid exposed to the fetus (Benediktsson et al., 1995). In supporting the view that the amount of maternal glucocorticoids affects offspring birth weight, it has been reported that a relative deficiency of term placental 11β -HSD2, with possible overexposure of maternal glucocorticoids to the fetus, is associated with reduced birth weight in human offspring (Stewart et al., 1995, Edwards et al., 1993). In addition, evidence from human studies shows that fetal overexposure to glucocorticoids, either by maternal treatment with synthetic

glucocorticoid dexamethasone (a poor substrate for 11 β -HSD2) or by maternal consumption of licorice, produces not only reduced birth weight, but also long-term alterations in HPA axis function, physiological, psychosocial and behavioural outcomes in children and adults (Doyle et al., 2000, Hirvikoski et al., 2008, Raikkonen et al., 2010).

A number of animal studies also provide evidence that prenatal administration of dexamethasone to pregnant dams can produce phenotypes similar to those in human studies, including low birth weight, hypertension, hyperinsulinemia, hyperglycemia, hyperactivity of the HPA axis, increased anxiety and altered cognition in the adult offspring (Nyirenda et al., 1998, Levitt et al., 1996, Brabham et al., 2000, Emgard et al., 2007, Welberg et al., 2001, Matthews, 2000, Hauser et al., 2006, Hauser et al., 2008). Conversely, maternal adrenalectomy and substitution with corticosterone (CORT) can reverse some of the long-term HPA axis and behavioural consequences induced by prenatal stress in the offspring (Barbazanges et al., 1996, Zagron and Weinstock, 2006).

Taken together, the above evidence lends support to the hypothesis that glucocorticoid programming is involved in mediating the link between prenatal stress and risk for adulthood diseases.

1.2.2 Accessibility of glucocorticoids to the developing fetus

One important consideration in the context of glucocorticoid programming is the accessibility of maternal glucocorticoids to the developing fetus. Under normal physiological conditions the fetus is protected from maternal glucocorticoids by the fetoplacenta barrier, which contains the enzyme 11 β -HSD2 that deactivates most of the maternal glucocorticoids (Seckl and Chapman, 1997). However this barrier is not complete, as it has been suggested that there is still 10-20% maternal glucocorticoids readily cross the barrier unconverted (Benediktsson et al., 1997). Thus, it is possible that excessive maternal glucocorticoids in response to stress-induced HPA axis activation may pass on to the fetus and interfere with fetal growth and development.

1.2.3 Clinical relevance

Glucocorticoids are prescribed for several conditions in the antenatal clinic. Synthetic glucocorticoids, dexamethasone or betamethasone, are used as a prophylactic treatment for pregnant women at risk of preterm labour (Liggins and Howie, 1972), and has been proven to substantially reduce neonatal mortality (National Institutes of Health Consensus Development, 2001). Glucocorticoids are also prescribed from early pregnancy if the fetus is at risk for congenital adrenal hyperplasia (CAH)(David and Forest, 1984). Long-term or repeated glucocorticoids administration is used in chronic conditions such as asthma or rheumatoid arthritis in pregnant women. Despite evidence of unequivocal clinical benefits, the potential risks related to antenatal use of glucocorticoids need to be evaluated (Fuchs et al., 2014).

There is evidence that antenatal glucocorticoid administration affects birth weight, length and head circumference in infant (Khan et al., 2011). In addition, there is increasing concern of the long-term consequences of antenatal glucocorticoid therapy. For example, it has been reported that late gestational glucocorticoid administration to mothers at risk for preterm labour leads

to long-term adverse cardiovascular outcomes in children and adults, including high blood pressure and hyperinsulinemia (Doyle et al., 2000, Dalziel et al., 2005). Early dexamethasone treatment for CAH has been associated with delayed psychomotor development (Lajic et al., 1998), psychosocial and behavioural problems in children (Trautman et al., 1995, Hirvikoski et al., 2008). Animal studies with rodents and non-human primates also indicate that fetal exposure to dexamethasone leads to abnormal development of physiology, motor, affective, cognitive and social behaviours in juvenile and adult offspring (Koehl et al., 2001, Matthews, 2000, Hauser et al., 2008).

Despite possible aversive effects of prenatal dexamethasone exposure indicated by these preliminary studies, no firm conclusions have been drawn about the potential risks related to antenatal glucocorticoid administration, especially about the life-long cognitive behavioural consequences. Given the fact that glucocorticoids are still widely prescribed in antenatal practice, it is important to gain more experimental data on the potential long-term neurobehavioural consequences of prenatal glucocorticoid exposure in offspring.

1.3 Animal models

In the context of the aforementioned safe use of antenatal glucocorticoids, animal studies provide indispensable tools to investigate the long-term neurobehavioral consequences of prenatal glucocorticoid overexposure, with the benefit of precise control and manoeuvre for experimental conditions, such that a correlation or causal relationship can be established. They further allow study of the neurobiological mechanisms that underlie behavioural phenotypes, with the potential of unravelling novel pharmacological therapies for treatment.

In this project, I used a rat model of dexamethasone (Dex) administered to pregnant dams during the last week of gestation. The construction of this animal model is based on the link between prenatal stress and increased risks of adulthood neuropsychiatric disorders from human epidemiological studies (detailed in 1.1) and its possible association with fetal overexposure to excessive maternal glucocorticoids (detailed in 1.2).

I will first discuss the construct validity of this animal model, with regards to the timing of exposure and glucocorticoids actions. I will then discuss the face validity of this animal model, by examining the phenomenological similarities between the reported phenotypes of this model and relevant clinical conditions.

1.3.1 Construct validity

I discussed the importance of timing in fetal programming in 1.1.3, it is thus crucial to consider the time of exposure when constructing an animal model. The animal model used in my current project is to give pregnant dams Dex injections during the last week of gestation, E15-21 inclusive. The rationale of choosing this time is firstly based on the evidence discussed in 1.1.3, such that stressors during the second trimester of human gestation is most strongly associated with the development of adulthood neuropsychiatric disorders. However there are

considerable differences in development timetables between humans and other mammalian species, and it has been suggested that the second stage of human gestation is mostly identical to E14-21 in rats in terms of brain development (Clancy et al., 2007). In addition, previous study shows that prenatal dexamethasone administration to pregnant dams induced programming effects on offspring physiology only when given during the last week of gestation, and no effect was observed when dexamethasone was given during the first or the second gestational week (Nyirenda et al., 1998). Another consideration is the accessibility of Dex to the fetal brain in rats. Dex is a poor substrate for 11 β -HSD2, which means it can readily cross the fetoplacental barrier (detailed in 1.2.2), and in a similar way bypasses the tissue specific 11 β -HSD2 expressed in the fetal brain. However the action of Dex on the fetal brain still depends on the local expression of its receptors. It has been suggested that Dex binds to glucocorticoid receptor (GR) at the experimental dose of 0.1mg/kg, and there is evidence that *GR* mRNA is expressed in the fetal brain from E12.5 in rats, whereupon its expression increases throughout gestation in different parts of the fetal brain (Diaz et al., 1998).

In summary, the construction of this animal model allows us to study the long-term behavioural and neurobiological effects of fetal overexposure to glucocorticoids, and to investigate the potential role of glucocorticoids in fetal programming of adult neuropsychiatric disorders.

1.3.2 Face validity

The phenomenological similarities between an animal model and relevant clinical conditions are measured by face validity, which is essential for extrapolating any knowledge derived from the model system to human conditions.

Several previous studies have investigated the cardiovascular and metabolic consequences of this model system, producing robust evidence of low birth weight, hypertension, and glucose intolerance in the adult offspring (Nyirenda et al., 1998, Levitt et al., 1996). These phenotypes are analogous to the cardiovascular and metabolic conditions that have been associated with prenatal stress in human patients (Khulan and Drake, 2012). Thus, this modelling proves a potential tool for studying some aspects of the cardiovascular and metabolic consequences of prenatal glucocorticoid programming.

In addition, it has been shown that this model system elicits neuroendocrine and behavioural phenotypes that resemble neuropsychiatric endophenotypes in the adult offspring, including HPA axis hypersensitivity, increased expression of fear and anxiety, reduced coping and learning in aversive situations (Weinstock, 1997, Welberg et al., 2001). The relevance of these phenotypes to clinical human conditions needs further validation, and the prenatal glucocorticoids effects on other aspects of cognitive function have not yet been explored.

In the current project, I aimed to further investigate the emotional and behavioural consequences of prenatal Dex exposure by using this rat model, and thus to investigate the face validity of using this model system for studying some of the related psychopathology changes in the context of prenatal glucocorticoid programming.

1.4 Epigenetics

Accumulating evidence suggests that an adverse early life experience is associated with increased susceptibility to diseases in adulthood, however the mechanisms of how these prenatal effects are established and sustained are not yet well understood. Recent studies suggest epigenetic mechanisms may provide a plausible link between early life adversity and sustained alterations in gene expression that lead to disease phenotypes in adults (Jirtle and Skinner, 2007).

The term ‘epigenetics’ was first coined by Conrad Waddington in the 1940s, in his study of the relationship between genes and their phenotypic products during embryological development (Waddington, 1968). He used this term to refer to things happening beyond the gene itself, addressing the fact that genetic variations do not always couple with phenotypic variations (Waddington, 1953). The concept has not changed much over the subsequent half a century, focusing largely on studies of cellular differentiation during embryonic development, of the temporal and spatial control of gene expression that gives rise to the various cellular phenotypes from the same genotype (Jablonka and Lamb, 2002). A real change in the scope of epigenetics took place when the molecular mechanisms controlling gene expression became clear, and when the dynamic nature of epigenetics began to be recognized. Epigenetics today refers to the mitotically and/or meiotically heritable, but reversible modifications of gene expression that are not encoded by the primary DNA sequence itself, encompassing a diversity of phenomena in addition to cellular differentiation, such as X-chromosome inactivation in females, repression of transposons and parent of origin specific expression of imprinted genes (Jablonka and Lamb, 2002, Goldberg et al., 2007).

1.4.1 Molecular mechanisms of epigenetics

There are several molecular mechanisms involved in epigenetics, including DNA methylation, histone modifications, non-coding RNA and chromatin conformation changes. Epigenetic modifications through these mechanisms can affect the expression of genes.

DNA methylation is by far the best-characterized mechanism of epigenetic regulation in the mammalian genome. It involves the transfer of a methyl group onto the 5'-position of the cytosine nucleotide, mostly in the context of Cytosine-phosphate-Guanine (CpG) dinucleotides at CG-rich gene regulatory regions. DNA methylation at the 5' promoter regions of genes is the most extensively studied mechanism of transcriptional silencing of genes, either by reducing the accessibility of transcription factors to their binding sites, or through recruitment of methyl-CpG binding domain-containing proteins (MBDs), which, together with histone modification complexes, lead to chromatin structure remodelling and subsequent transcriptional repression (Nan et al., 1998).

Histone modifications including acetylation, methylation, phosphorylation ubiquitination and sumoylation are also involved in transcriptional control of gene expression by affecting chromatin accessibility (Bannister and Kouzarides, 2011). Non-coding RNAs (ncRNAs), including long ncRNAs (lncRNAs) (longer than 200 nucleotides) and short ncRNAs (<200 nucleotides long), have also been implicated in various epigenetic regulatory processes (Mattick and Makunin, 2006, Zhou et al., 2010). For example, there is evidence that lncRNAs are involved in recruiting DNA methyltransferases (DNMT), histone modifying enzymes and chromatin remodelling complexes to their sites of action (Zhou et al., 2010). Short ncRNAs, including microRNAs (miRNAs) and small interfering RNAs (siRNAs) are also involved in epigenetic regulation of gene expression through transcriptional silencing and post-

transcriptional silencing, such as translational repression and RNA interference (Chu and Rana, 2007).

1.4.2 The dynamic nature of epigenetics

Epigenetic marks are thought to be established during development and subsequently retained through mitosis, promoting cellular identity in future cell generations. For a long time, these epigenetic marks have been regarded as predominantly irreversible, however in recent years it has been recognized that epigenetic marks are dynamic and continuously responsive to genetic, environment and stochastic factors even in fully differentiated mature cells (Feil and Fraga, 2011). I here discuss evidence that epigenetic marks are modifiable during embryogenesis and through adulthood, and that they may serve as an interface between genetic traits and environmental factors.

1.4.2.1 Embryogenesis

During embryogenesis, the epigenetic signatures of the mammalian genome (epigenome) undergo precise, coordinated reprogramming at distinct stages of development (Seisenberger et al., 2013).

Mouse studies suggest there are two global DNA demethylation and subsequent re-methylation events during mammalian development. The first wave of DNA demethylation happens immediately following fertilization in the zygote, with important exceptions in imprinted genes, active retrotransposons and heterochromatin regions, for the benefit of maintaining parental imprinting, transposon repression and chromosomal stability, respectively (Seisenberger et al., 2013). Remethylation follows, progressively generating epigenetic marks that reflect cellular lineage decisions and the developmental history of the embryo (Seisenberger et al., 2013).

In order to give rise to gametes, the primordial germ cells (PGCs) undergo a second wave of DNA demethylation to erase their somatic epigenetic patterns. The erasure of DNA methylation in PGCs appears to start from approximately E8.0 and completes by approximately E13.5 (Rose et al., 2013). The following wave of remethylation is sex-specific. It is believed to initiate at around E15 in male germ cells and occur after birth in the growing oocytes in female germ cells (Rose et al., 2013). This second wave of epigenetic reprogramming resets most DNA methylation marks including parental imprints to reflect the gender of the embryo.

These developmental reprogramming events are characterized by remarkable changes of epigenetic marks, which may render these time points more susceptible to environmental perturbations compared to the fully differentiated state. Evidence of environmental influence on the epigenome during development is discussed below in 1.4.3.

1.4.2.2 Adulthood

Although embryogenesis represents a time of increased vulnerability, the environment can impact on the epigenome in postnatal life as well. Direct evidence comes from studies in monozygotic twins, who were found to be epigenetically indistinguishable during the early years of life, but who developed remarkably different epigenetic traits in their older age (Fraga et al., 2005). In adults, the dynamic nature of the epigenome is evident in a variety of physiological processes such as hippocampal neurogenesis, neuroplasticity and memory formation. In addition, there is direct evidence that various environmental factors such as toxins, drugs and chronic stress can induce epigenetic changes in healthy subjects, as detailed below.

Adult neurogenesis

In adults, there are still thousands of new neurons generated from neural stem cells in discrete regions of the mammalian brain every day. Adult neurogenesis, the generation of functional neural cell types from adult neural stem cells, is dynamically regulated by epigenetic mechanisms and is in response to a variety of internal physiological, environmental and experience-dependent clues (Ma et al., 2010).

Neuronal plasticity and memory formation

Epigenetic mechanisms are implicated in experience-dependent learning and memory formation (Day and Sweatt, 2011). For example, it has been reported that DNA methylation and histone acetylation are dynamically regulated in the adult brain in response to experience (Levenson et al., 2006), and that they are involved in the induction of synaptic plasticity and memory formation and maintenance (Day and Sweatt, 2010, Levenson et al., 2004, Levenson et al., 2006). There is also evidence that environmental factors such as stress and hormones can influence epigenetic processes, leading to altered neural function and memory formation (Feng et al., 2007).

Exposure to environmental factors

Increasing evidence suggests that exposure to various environmental toxicants can induce phenotypic changes in epigenetic patterns during the lifetime. For example, tobacco smoking has been associated with altered DNA methylation at promoter regions of several tumour suppressor genes in the bronchial epithelial cells (Belinsky et al., 2002). Global DNA methylation changes were found in blood cells of individuals exposed to the carcinogen benzene (Bollati et al., 2007) and air pollutants (Baccarelli et al., 2009). Chronic exposure to sunlight has been shown to induce epigenetic changes in skin cells (Gronniger et al., 2010). Epigenetic changes in brain reward regions have been implicated in the pathogenesis and persistence of drug addiction (Renthal and Nestler, 2008). Likewise, exposure to chronic stress

is believed to induce phenotypic epigenetic changes across multiple generations (Skinner, 2014).

In conclusion, the dynamic nature of the epigenome can be seen in a variety of physiological and pathological processes along the history of life, and epigenetic traits may be modifiable and responsive to environmental stimuli irrespective of the stage of development.

1.4.3 Epigenetics and programming

As discussed in 1.4.2.1, the developmental period is a time of particular vulnerability to epigenetic perturbations, as active changes in epigenetic marks are taking place as part of developmental reprogramming and subsequent cellular lineage differentiation. Environmental influence on the epigenome during development can be maintained through subsequent cell divisions, and thus induce an enduring impact on later life.

1.4.3.1 Prenatal effects

A number of animal studies and human data have documented that prenatal environmental factors, such as maternal diet, maternal exposure to stress and maternal glucocorticoids administration during pregnancy can modulate the epigenetic signatures of the offspring, accompanied by sustained alterations in gene transcription and phenotype.

Diet

It has been reported that exposure to a low-protein diet during pregnancy in rats leads to altered epigenetic regulation and gene expression of the peroxisomal proliferator-activated receptor (*Ppar*) gene, which encodes a product involved in lipid homeostasis, in the liver of rat offspring after weaning (Lillicrop et al., 2005). Similarly, a maternal low-protein diet has been associated with epigenetic silencing of the hepatic nuclear factor 4 α (*Hnf4 α*) gene with

associated decrease in gene expression in the pancreatic islets of rat offspring, which has been linked to the development of type 2 diabetes in the same animal model (Sandovici et al., 2011). In addition, dietary restriction during early development has been found to cause widespread alterations in DNA methylation in the fetal liver in sheep, associated with altered immune responses to antigenic challenge, insulin-resistance and elevated blood pressure in adults (Sinclair et al., 2007). Studies on the Dutch hunger cohort have provided the first human evidence that maternal famine during early gestation leads to persistent changes in DNA methylation at genes related to growth and metabolic diseases, such as insulin-like growth factor 2 (*IGF2*), insulin (*INS*), guanine nucleotide binding protein α -stimulating (*GNAS*) and maternally expressed gene 3 (*MEG3*) (Tobi et al., 2009, Heijmans et al., 2008).

Maternal stress

There is evidence that prenatal stress induces epigenetic changes on a number of candidate genes likely to be implicated in neuropsychiatric disorders, including glutamic acid decarboxylase (*GAD67*), reelin and brain-derived neurotrophic factor (*BDNF*). One study reported that maternal restraint stress in mice leads to overexpression of DNA methyltransferase 1 (*Dnmt1*) in GABAergic neurons, resulting in hypermethylation at the promoter regions of *Gad67* and reelin, associated with decreased mRNA expression of relevant genes in early and adult life (Matrisciano et al., 2013). Similarly, another study reported that prenatal stress leads to decreased cortical expression of reelin in rat fetuses, accompanied by hypermethylation at the gene promoter region. These molecular changes are associated with persistent behavioural consequences in adults, including increased spontaneous locomotor activity, higher anxiety levels and cognitive deficits (Palacios-Garcia et al., 2015). A recent study also reported decreased expression of *Bdnf* transcripts in the prefrontal cortex and hippocampus of prenatally stressed mice, associated with increased expression of *Dnmt1* and hypermethylation at the *Bdnf* gene regulatory regions (Dong et al., 2015). In another study, researchers found increased levels of DNMT1 protein in the

hippocampus of prenatally exposed mice, associated with impaired performance in water maze test (Benoit et al., 2015). In addition, evidence from human studies indicated that maternal stress during pregnancy is associated with DNA methylation changes at differentially methylated regions (DMRs) of imprinted genes, including mesoderm specific transcript (*MEST*) (Vidal et al., 2014), *MEG3* and *IGF2* (Liu et al., 2012).

Glucocorticoid exposure

It has been reported that Dex administration during the last week of gestation in rats is associated with decreased DNA methylation at one of the DMRs of *Igf2* in the fetal liver (Drake et al., 2011). Studies with guinea pigs also suggested that glucocorticoids treatment during late gestation induces genome wide changes in promoter DNA methylation and histone acetylation in the fetal hippocampus (Crudo et al., 2013). The same group of researchers also reported changes in the developmental trajectory of DNA methylation in several other fetal tissues of prenatally exposed guinea pigs, associated with altered expression of genes involved in DNA methylation process (Crudo et al., 2012).

1.4.3.2 Postnatal effects

After birth, the prepubertal brain still undergoes remarkable changes in both structure and function and is therefore vulnerable to remodelling by environmental factors (Romeo and McEwen, 2006). There is evidence that the impact of postnatal experience on brain function may be partly mediated by long-lasting epigenetic changes in the offspring, as discussed below.

The first report in this field was made by Weaver by showing that low levels of maternal care in rats is associated with hypermethylation at regulatory regions of *GR* (also known as *Nr3c1*), leading to reduced binding of a transcription factor, nerve growth factor inducible gene-A

(NGFI-A), and subsequent decrease in hippocampal *GR* expression (Weaver et al., 2004). Similar findings were reported in humans, for example childhood abuse has been associated with hypermethylation at NGFI-A binding site of the *NR3C1* promoter region, with associated reduction in hippocampal *GR* expression in adult suicidal victims (McGowan et al., 2009).

Possible implications of other candidate genes have also been reported, including genes involved in the HPA axis regulation, genes associated with cognitive and emotional behaviour and genes involved in epigenetic processes. For example, one study reported early life stress causes persistent hypomethylation at an important regulatory region of the arginine vasopressin (*Avp*) gene in the paraventricular nucleus (PVN) of exposed mice, with functional and phenotypic relevance of increased *Avp* expression, increased HPA axis activity and behavioural alterations (Murgatroyd et al., 2009). Another study reported site-specific hypermethylation at the *Bdnf* promoter region, with functional relevance of decreased *Bdnf* expression in the prefrontal cortex of rat offspring of chronically stressed mothers (Roth et al., 2009). In another study, maternal separation leads to altered DNA methylation patterns in the promoter regions of several candidate genes in the germline cells of mice offspring, including methyl CpG-binding protein 2 (*Mecp2*), cannabinoid receptor-1 (*Cnr1*) and corticotrophin-releasing hormone receptor 2 (*Crhr2*). These changes in methylation are functionally related to altered levels of mRNA expression, and can be maintained and passed on to the next generation (Franklin et al., 2010). Another recent study also reported that mild adolescence separation stress in mice with genetic risks leads to DNA hypermethylation of the tyrosine hydroxylase (*Th*) gene in dopaminergic neurons, associated with several neurochemical and behavioural deficits in offspring (Niwa et al., 2013).

In addition, there is also evidence that the impact of postnatal experience on DNA methylation is both genome-wide and system-wide. For example, one study reported that different postnatal rearing conditions lead to genome-wide alterations in promoter methylation in the

prefrontal cortex of rhesus monkeys. DNA methylation changes are not limited to the brain, but are also evident in the peripheral T cells (Provencal et al., 2012). Similarly, childhood abuse is associated with genome-wide changes in promoter methylation in the adult hippocampus of suicidal victims, with functional relevance of altered gene expression involved in cellular/neuronal plasticity (Labonte et al., 2012).

In summary, these studies provide important evidence that epigenetic changes are involved in the long-term pathological trajectories predisposed by early life environmental events, lending support to the hypothesis that epigenetic mechanisms may be implicated in the fetal programming of adult diseases.

1.4.4 Epigenetics and neuropsychiatric disorders

Important efforts have been made to elucidate the molecular basis underlying human neuropsychiatric conditions. Increasing evidence suggests that epigenetic mechanisms are involved in mediating the long-lasting alterations in brain function in neuropsychiatric disorders (Tsankova et al., 2007).

Direct evidence supporting the role of epigenetics in neuropsychiatric disorders comes from genome-wide scans which revealed significant changes in DNA methylation status of single loci candidate genes as well as global changes in epigenetic landscapes between psychiatric patients and healthy controls, using DNA samples extracted from human post-mortem brain and blood leukocytes samples. Evidence was found in an array of neuropsychiatric disorders, including schizophrenia (Grayson et al., 2005, Carrard et al., 2011, Liu et al., 2014), ASD (Nguyen et al., 2010) and bipolar disorders (Mill et al., 2008, Carrard et al., 2011). In addition, epigenetic-related processes, such as adult neurogenesis (Hsieh and Eisch, 2010), retrotransposition and genomic imprinting (detailed in chapter 6), have been implicated in

neuropsychiatric disorders, providing further evidence that epigenetic mechanisms may be involved in the pathogenesis of neuropsychiatric disorders.

Detailed reviews of our current knowledge regarding the epigenetic mechanisms involved in common neuropsychiatric disorders such as ASD, schizophrenia and bipolar disorders can be found in some good reviews (Labrie et al., 2012, Siniscalco et al., 2013). Together with existing evidence of epigenetics in programming (discussed in 1.4.3), it is very likely that epigenetic regulatory mechanisms may represent a link between prenatal environmental factors and subsequent long-term impact on mental health in adult life.

1.5 Hypotheses and aims

I hypothesise that intrauterine overexposure to glucocorticoids will induce programming effects on brain development and function in the offspring, the effects of which may endure into adult life and affect later mental health.

This study is aimed at exploring possible effects of prenatal glucocorticoid overexposure on the functioning of the brain and to investigate possible molecular mechanisms underlying the prenatal glucocorticoid programming effects. By using a rat model of prenatal Dex treatment during the last week of gestation, I will explore the effects of prenatal glucocorticoid overexposure on fetal growth as well as the long-term programming effects on stress response and cognition. I will also explore the molecular basis underlying the behavioural phenotypes of the prenatal Dex exposure model by studying the expression of candidate genes known to be involved in relevant behaviour. Finally, considering the plausible role of epigenetics involved in prenatal programming, I will therefore study the effects of prenatal glucocorticoid overexposure on brain expression of candidate genes involved in two epigenetically regulated processes such as retrotransposon activities and genomic imprinting. An outline of experiments involved in this thesis is listed below.

1.6 Outline of experiments

The content of my current study include:

Chapter 3

Basal and stress-induced plasma CORT levels

Elevated plus-maze

Open field

Contextual fear conditioning

HPA axis activity

Unconditioned fear and anxiety-related behaviour

Unconditioned fear and anxiety-related behaviour

Unconditioned fear and anxiety-related behaviour

Contextual fear conditioning and extinction

Chapter 4

Spatial reference memory with reversal learning

Delayed matching to position (DMTP)

Intra/Extra-dimensional (ID/ED) attentional set-shifting

Spatial reference memory

Within-task behavioural flexibility

Working memory

Within-task behavioural flexibility

Behavioural flexibility within attentional settings

Chapter 5

Brain gene expression

Candidate genes involved in behaviour

Validation of next generation RNA Sequencing (RNA-Seq) data on adult prefrontal cortex (PFC) samples

DNA methylation at CpG-rich regions of *Arc* gene

Possible epigenetic regulation of *Arc* transcription

Chapter 6

Brain gene expression

Candidate genes including retrotransposons and imprinted genes

DNA methylation at DMRs of imprinted *Dlk1-Dio3* and *Igf2* regions

Prenatal glucocorticoid effects on DMRs DNA methylation

Chapter 2 Materials and methods

2.1 Buffers and Solutions

Room temperature (RT°) is defined as 18-25°C.

0.5M EDTA (pH 8.0): Disodium ethylenediaminetetraacetate.2H₂O (186.1g) was dissolved in 800ml of Milli-Q water. pH was adjusted to 8.0 by adding 10M KOH and the final volume was adjusted to 1L with Milli-Q water. The buffer was sterilized in an autoclave and kept at RT°.

DEPC-treated water: 7 drops of Diethyl pyrocarbonate (DEPC) was added to 500ml of Milli-Q water. The container was vigorously shaken and left at RT° in the fume hood overnight before being autoclaved.

10x TBE Buffer: Tris base (108g), boric acid (55g) and 40ml of 0.5M EDTA were dissolved in 800ml of Milli-Q water. pH was adjusted to 8.0 by adding 10M KOH and the final volume was adjusted to 1L with Milli-Q water. The buffer was autoclaved and kept at RT°.

0.5x TBE Buffer: 50ml of 10x TBE was diluted in 950ml of Milli-Q water and stored at RT°.

Chaos Buffer: Guanidinium thiocyanate (53.2g), N-lauroylsarcosine (sarcosyl) (2.0g) and 10ml of 0.5M EDTA (pH8.0) were dissolved in DEPC water, and the final volume was adjusted to 100ml. The buffer was filtered and kept at RT°. 0.1M 2-mercaptoethanol (14.33 stock solution 693ul) and 0.2% antifoam-A (concentrated solution 200μl) was added before use.

2.2 Animal maintenance

All animal procedures were carried out under project license number 60/3962 and 70/7870 and personal license number 60/13373 under the terms of the Animals (Scientific Procedures) Act 1986. William Mungall and Richard Watson were responsible for the daily maintenance of rats in the Biomedical Research Facility (BRF) Little France facility and BRF 1 George Square facility, respectively. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and were conducted strictly according to local ethics guidelines to minimize animal suffering and the number of animals to be used.

Wistar rats were bought from Charles River UK. Female and male rats were purchased for mating at 2 months old. Animals had *ad libitum* access to food (standard rat chow, RM1, Special Services Diet, Lillico, Surrey, UK) and water. Rats were maintained under controlled temperature (between 19 and 21°C) and light/dark cycle (lights on from 07:00 to 19:00) in both units. Rats were kept at a maximum of five per cage and cages were cleaned weekly.

Rats were allowed to acclimatize for two weeks after arrival at the BRF. At mating, a female was housed individually with a male of similar age. Following vaginal plugging (denoted as E1) pregnant dams were randomly assigned to Dex or vehicle (Con) treatment. From E15-E21 inclusive, dams were given a daily subcutaneous injection of either Dex (100µg/kg body weight, dissolved in 4% ethanol, 0.9% saline) or an equivalent volume of vehicle (4% ethanol, 0.9% saline) (0.5ml/kg). At birth (postnatal day 1), the offspring (Dex or Con) were weighed, sexed and litters culled back to eight per group, leaving 5 males and 3 females per litter where possible. Pups were reared by their biological mothers until weaning at three weeks of age, whereupon female offspring were culled and male pups were housed in same treatment groups. Animals were then left undisturbed until adulthood.

2.3 Molecular procedures

2.3.1 Tissue collection

Animals from each group were taken from their home cage and killed by exposure to a rising concentration of carbon dioxide followed by decapitation following loss of consciousness. Brains were immediately removed and immediately dissected into five parts, namely prefrontal cortex (PFC), hippocampus, hypothalamus, cerebellum and the rest of brain. For adult brain tissues, each part of the brain was kept separately as left and right. Dissected tissues were snap frozen on dry ice and stored at -80°C.

2.3.2 Simultaneous RNA/DNA extraction

Total genomic DNA and RNA were extracted from individual dissected brain samples. Approximately 30mg of frozen brain tissue was used for the simultaneous extraction of total genomic RNA and DNA. Each individual sample was homogenized in short bursts in 800µl Chaos Buffer. The homogenate was immediately divided in half and used for RNA or DNA extraction.

RNA extraction was conducted immediately after homogenization to avoid degradation. 40µl 2M sodium acetate, 400µl acidic phenol (pH4.2) and 200µl chloroform/isoamyl-ethanol were added to the homogenate. Samples were incubated on wet ice for 10 min and then centrifuged at full speed for 20 min at 4°C. An equal volume of 100% isopropanol was added to the upper phase, and the mixture was then transferred to a Qiagen RNeasy spin column (RNeasy® mini kit, Qiagen). All samples were then washed following the manufacturer's protocols and were eluted in a final volume of 30µl RNase-free water. For adult PFC samples prepared for next generation sequencing, a RNeasy® plus mini kit was used (Qiagen) and manufacturer's protocols followed.

The allocated portions for DNA extractions were kept on wet ice while waiting for the RNA extraction to be conducted. 300µl AL Buffer and 300µl 100% isopropanol were added to the homogenate, and the mixture was then transferred to a DNeasy mini spin-column (DNeasy® Blood and Tissue kit, Qiagen). DNA extractions were conducted following the manufacturer's protocols. 5µl RNase A (invitrogen) was added to the membrane and let sit at RT° for 10 min after adding 500µl of buffer AW1. All samples were then washed following the manufacturer's protocols and were eluted in a final volume of 200µl buffer AE for 10 min at 70°C.

2.3.3 Quantification and quality check

RNA/DNA concentrations were assessed by using the Qubit® RNA HS Assay Kit and Qubit® dsDNA HS Assay Kit, respectively. Manufacturer's instructions were followed. All RNA/DNA samples were visualized on a 1% agarose gel containing 1:10 GelRed™. Approximately 2µl of extracted RNA/DNA were mixed with 3µl of loading buffer (Orange G). A 1 kb DNA ladder was loaded along with samples to estimate size and to act as control. The gel was run at 110 volts for 45-50 minutes and viewed using a Gel-doc system at 260nm. The integrity of RNA was indicated by the presence of clean thicker 28S and thinner 18S ribosomal RNA bands (at about 2:1 ratio), and without a DNA band. A thick DNA band without any RNA smears was considered good quality DNA.

2.3.4 Gene expression analysis

2.3.4.1 Reverse transcription of RNA

After Qubit quantification (as described in 2.3.3), RNA samples were diluted to a concentration of 100ng/μl, and 8μl of each was used for reverse transcription (RT) reaction. DNase treatment was performed prior to RT reactions following the Promega protocol. After DNase treatment, RT was conducted by using the high capacity cDNA reverse transcription kit (Applied Biosystems, UK) and the manufacturer's instructions were followed. For each sample, a master mix consisting of: 10x RT buffer (2.0μl), 25x dNTP Mix (0.8μl), 10x RT Random Primers (2.0μl), RNasin inhibitor (1.0μl), MultiScribe™ reverse transcriptase (1.0μl) was added, and the total volume was adjusted to 20.0μl by adding Nuclease-free water. A – RT (no reverse transcriptase) sample was made: the sample underwent all the procedures mentioned above except that nuclease free water was added in place of AMV reverse transcriptase. This acts as a negative control to ensure no genomic DNA contamination occurred. All samples were then reverse transcribed following the manufacturer's instructions: at 25°C for 10 minutes followed by 37°C for 2 hours, then 85°C for 5 minutes then gradually cooled down to 4°C in a G-Storm thermal cycler. The RT products, complementary DNA (cDNA), were stored at -20°C.

For cohort 1 cDNA samples, I used 12ul of each sample for dilution 1 in 20, the final volume of 240ul of each diluted sample were aliquot into two strip sets. The remaining 8ul of cDNA from each sample were pooled together and series dilutions were performed (standard curve strips are labelled in red). For cohort 2 cDNA samples, I used 13ul of each sample for 1 in 20 dilution, the final 260ul diluted cDNA were aliquot into three strip sets. The remaining 7ul of cDNA from each sample were pooled together and stored in one tube (labelled in blue ink as pooled cDNA), which can be used to make standard curves when needed.

2.3.4.2 Quantitative real-time polymerase chain reaction (qPCR)

The quantity of mRNA was measured by qPCR. Individual samples of cDNA were diluted 1:20 in nuclease free water. Samples were run together with a standard curve made by pooling cDNA from all the samples and serially diluted in nuclease free water (1:4, 1:8, 1:16, 1:32, 1:64, 1:128). A –RT control and a non-template control (nuclease free water) were also run at the same time to ensure no contamination occurred.

For each assay, 8µl of master mix containing corresponding primers, probe and Roche® probes master were loaded into the bottom of a 384 well plate in triplicate (Table 2.1). UPL assays were self-designed using the Universal Probe Library™ (UPL) system (Roche Diagnostics Ltd., West Sussex, UK). All self-designed UPL assays were intron-spanning assays. PrimeTime® qPCR primers and probes were designed by Dr. Jessy Cartier using the Integrated DNA Technologies system. ABI assays were bought from Taqman® as primer-probe mix.

After the loading of master mix, 2µl of samples, negative controls and serial dilution of standard curve samples were then loaded into the wells in triplicate for each assay. Plates were centrifuged for 2 minutes at 1500 revolutions per minute (rpm) and then loaded into the LightCycler® 480. The reaction conditions comprised rapid heating to 95°C for 5 minutes for denaturation followed by 50 cycles of a denaturation step at 95°C for 10 seconds and annealing at 60°C for 30 seconds. The samples were then cooled to 40°C for 30 seconds for completion. Acceptable assays had a reaction efficiency value between 1.7-2.1. Data were analysed using generalised linear models (JMP statistical software, SAS Institute, Cary, NC, USA). All genes of interest were normalized to housekeeping genes.

	Self-designed primer	Taqman® primer- probe
Roche Probes Master	5µl	5µl
Taqman® primer-probe mix	-	0.5µl
Forward primer	0.02µl (100µM)	-
Reverse primer	0.02µl (100µM)	-
Probe	0.1µl (10µM)	-
cDNA	2µl	2µl
Nuclease free water	2.7µl	2.5µl

Table 2.1 Recipes for qPCR with self-designed UPL/PrimeTime® primers or Taqman® primer-probe mix.

2.3.4.3 Rat self-designed UPL primers

Primer	Forward Primer	Reverse Primer	UPL Probe No.	Amplicon (nt)
Arc	gctgaagcagcagacctga	tctgcttttctcactggatga	3	74
Bdnf	gcagtcagtgcctttggag	cggcatccaggaatttttg	65	69
Calcr	gtgctatgaccggattcagc	ccaagtcggttgcaatatg	7	67
Egr1	cgaacaaccctacgagcac	gcgccttctcgttattcaga	114	68
G6pd	gcaaacagagtgagcccttc	tggctgttgaggtgcttg	75	130
Gfral	cgaatgatgaattatgcagga	caggtctcatcttcacgacact	5	92
Gnas	ttcgagtggttggaagc	accaagaagccgactcacc	21	76
Gpr101	gaaagtggtgccagacagc	cagctcaatgtctgccctagt	17	122
Hprt	gaccggttctgtcatgtcg	acctggtcatcatcactaatcac	95	61
Isl1	gcaaccaacgacaaaactaat	ccatcatgtctctccgact	83	78
Ngfr	acactgagcgccagttacg	catcgaccagggatctcttc	26	70
Nts	tgactctctggctttcagc	gcctccaggactctcacatc	109	70
Qrfpr	cgtgcaacgccttgagat	aagtgctgtagattctctggtg	84	105
Rspo1	cgacatgaacaaatgcatca	ctcctgacacttggtgcaga	5	76
RBII85	ctgaggatacctgaagtgaagg	caacttcattgatgtccatactc	6	
Tbp	cccaccagcagttcagtagc	caattctgggttgatcattctg	129	75
Th	cggagctgattgcagaga	agcgtgacatatacctcctcc	97	110

2.3.4.4 Rat self-designed PrimeTime® primers

Primer	Forward Primer	Reverse Primer	Probe	Position
ORF2	ccttcatagaagacctcataccaata	tgttgtgtctttgtgtggttag	tgaacagatggagccctaccgaa	Universal
Rat1	ccagctcaaaggaccagtaaa	ttatgtgacgggaggtgttc	tggagtctgtaggcttctgtatgcc	Chromosome 1
Rn1	ctgaaggaaacagaccggataa	ggagcagtctcttctggtttc	agttctctgcacccaaatcccgt	Chromosome 11

2.3.4.5 Rat Taqman® primer-probe set

Primer	Accession Number	Amplicon (nt)
Cdkn1c	RN00711097_m1	70
Dio3	RN00568002_s1	63
Dlk1	RN00587011_m1	74
GR (Nr3c1)	RN01405584_m1	75
Grb10	RN01401196_m1	101
Igf2	RN01454518_m1	74
Snrpn	RN00579591_m1	80
Ube3a	RN01409766_m1	65

2.3.5 Pyrosequencing

2.3.5.1 Bisulfite conversion

After Qubit quantification (as described in 2.3.3), 1µg DNA was made up to 45µl in a 200µl PCR tube. All samples were bisulfite converted using an EZ DNA Methylation™ Kit (Zymo Research, USA). After the addition of CT conversion reagent, incubation was performed in a G-Storm thermal cycler overnight. The incubation conditions comprised rapid heating to 95°C for 5 minutes followed by 16 cycles of 95°C for 30 seconds, 50°C for 60 minutes and then hold at 4°C. All samples were then washed following the manufacturer's protocols and the final products were eluted in 30µl of M-Elution Buffer and stored at -20°C.

2.3.5.2 Polymerase chain reaction (PCR)

PCR primers were self-designed for the promoter and gene body regions of *Arc* using the PyroMark Assay Design 2.0 software (Qiagen). *Igf2*-DMR2 and IG-DMR methylation was analysed using assays from Dr. Khulan Batbayar. All primers were purchased from Applied Biosystems, UK. Primer sequences are listed in 2.3.5.4.

For each assay, 1µl of bisulfite converted DNA was used as template in a 20µl PCR reaction mixture with the addition of primers and AmpliTaq® Gold PCR Master Mix (Applied Biosystems). The amount of primers being used was optimized for each assay. A non-template control (nuclease free water) was run at the same time to ensure no contamination occurred. PCR products were electrophoresed on a 1% agarose/0.5 × TBE gel containing 1:10 GelRed™ at 110V for 45 minutes. A 100bp DNA ladder was loaded along with samples to estimate size and to act as control. Gels were visualized under UV light using a Gel-doc system at 260nm. A sharp contrast between the strength of product band and any primer-dimer band was considered satisfactory. The optimized PCR program for all assays comprised rapid heating to 95°C for 10 minutes for denaturation followed by 45 cycles of a denaturation at 95°C for 20

seconds, annealing at 56°C for 20 seconds and elongation at 72°C for 20 seconds. The samples were then stored at 72°C for 7 minutes for completion.

2.3.5.3 Pyrosequencing

Pyrosequencing was carried out using the PyroMark Q24 system (Qiagen). 15µl of PCR products from 2.3.5.2 was added along with 2µl of streptavidin-coated sepharose beads (GE Healthcare Life Sciences, Sweden) and 40µl binding buffer to a 24-well plate. The volume was adjusted to 80µl using Milli-Q purified water. Two negative controls (one non-template PCR product and one sequencing primer) were loaded on the same plate to ensure no contamination occurred. Plates were sealed and agitated for 5 minutes at 14000 rpm. Samples were captured using a Pyromark suction device and flushed stepwise following the manufacturers' instructions. The beads were then released into a sequencing primer plate prepared by loading 0.3µM sequencing primer in 25µl of annealing buffer. This plate was then incubated at 80°C for 2 minutes and cooled down to RT° for 5 minutes to allow the annealing of sequencing primers to templates. Appropriate volumes of enzyme, substrate and dNTPs (PyroMark Q24 Gold Reagents, Qiagen) were added to the PyroMark Q24 cartridge as indicated by the PyroMark Q24 analysis software. The plate was processed by the Pyromark Q24 instrument by fitting in the filled cartridge and starting the program, and was then analysed by the PyroMark Q24 analysis software.

2.3.5.4 Pyrosequencing PCR primers

Gene	Forward Primer	Reverse Primer (5'-Biotinylated)	Sequencing Primer	Amplicon length
<i>Arc</i> , promoter, product 1 (<i>Arc_P1</i>)	TGTGAAGGGGAGGGTTTTTGA G	ACCCTAACTCCCATTAACC	GGGAGGGTTTTTGAGGG	133
<i>Arc</i> , promoter, product 2 (<i>Arc_P2</i>)	GGGTTTGAGGTAGGAGAGGG	AAAAAACTACTCCCCAAAACA CTACACT	GGGAGAGGTTAATGGG AGTTA	157
<i>Arc</i> , gene body, product 1 (<i>Arc_GB1</i>)	TTTTTTGGTTTTAGTTTTAGAG GAGTT	CCTATACAACCCTTTCAACTCT	GTAGATGGAGTTGGAT	260
<i>Arc</i> , gene body, product 2 (<i>Arc_GB2</i>)	GGTGGGTAAGTTGGAGAATAA T	CCTCTCCAAACCATAAAAAAC CTC	TTGGAAGAAGTTTATTA AGG	175
<i>Igf2</i> -DMR2	GTTGTTGGATATTTTTGAAGA GGTT	CCTACTAACTAACACCTCCTCT CC	GGGTTTTTGGGTGGTAA TA	

IG-DMR1, (IG-DMR1.2)	TTGGAAAGTGTAGTGAGGTGA	ACCTTCAACCTATAAAATAAA	GTTTATTAAGGTTTGGT
	TAG	ACTTTAC	T
IG-DMR1, (IG-DMR1.3)	AGGTTAAGAGGTTTGTGGTTT	ACACCCAACTTATACCATACAT	AGTGTTTTATAGTATAT
	AAGTA		AGATAAG
IG-DMR2, (IG-DMR2.2)	TGGTATAGATAGATGGTATAT	TCCCATAACACAAATACACAA	ATAATGGATTTTGTATT
	TGTATAGGA	AATACTACA	TGTA
IG-DMR2, (IG-DMR2.3)	TTGTGTATTTGTGTTATGGGAT	CTTATACACAATAAACCCCTAA	TTTATATTGTGTATAAT
	ATTTTA	AACTAACC	GTTTTTG
IG-DMR2, (IG-DMR2.4)	ATGTTTGTATTATGGTGTTTTA	AATCCTTATACACAATAAACC	GTGTTGTGGATTTAGGT
	TGTAGTTA	CTAAAACTA	
IG-DMR3, (IG-DMR3.2)	AGTGTTGTGGGTGTATTTTAG	CTCCCCCTTAAAAAAAATTACC	AGTTTGATTGTAGTTTTT
	AT	CAACCCT	ATATTAT

**Chapter 3 Glucocorticoid programming:
effects on stress response in adult rats**

3.1 Introduction

3.1.1 Introduction

Fear and anxiety are important emotions. Fear (towards a real, current threat) and anxiety (towards an anticipated threat) evoke a stress response in the body, which involves not only the immediate physiological and behavioural responses to combat the threats, but also an appropriate extinction of the response to maintain internal homeostasis and subsequent adaptive behaviour. An inappropriate stress response can express as hypersensitivity to an acute stress, and/or a failure to consolidate the memory and make adjustments for future behaviour. A number of animal studies have provided evidence that prenatal stress leads to physiological and behavioural changes that represent a maladaptive stress response, suggesting that prenatal stress may induce susceptibility to stress-related psychopathological changes in later life (Koehl et al., 2001).

The physiological activation of the HPA axis system is intimately linked to a stress response. Plasma CORT levels are key indicators of HPA axis function. Despite reports that prenatal stress leads to elevated basal plasma CORT levels (Green et al., 2011b), the dysfunction of the HPA axis induced by prenatal stress seem to predominately involve a failure to attenuate the stress response via feedback mechanisms, demonstrated by a heightened or prolonged elevation of stress hormones after an acute stress (Weinstock, 1997, Henry et al., 1994, Weinstock et al., 1992, Wilson et al., 2013, Maccari et al., 1995, Vallee et al., 1997). There is also evidence that prenatal stress affects the time-course of HPA hormone fluctuations after repeated exposure to stress (Fride et al., 1986).

Fear and anxiety can also be assessed by behavioural reactions. Several behavioural measures are now available allowing for assessment of different aspects of fear and anxiety, including unconditioned fear, fear potentiation, fear consolidation and fear extinction. Offspring of

stressed dams have been found to show increased fear and anxiety-related behaviour in a stressful environment, including increased aversion to the open arms on an elevated plus-maze (Wakshlak and Weinstock, 1990, Vallee et al., 1997, Welberg et al., 2001, Estanislau and Morato, 2005, Bondi et al., 2008, Poltyrev et al., 1996a), decreased exploration and increased defecation in an open field (Poltyrev et al., 1996a, Welberg et al., 2001) and increased defensive freezing (Takahashi et al., 1992). In addition, there is evidence that prenatal stress leads to impaired habituation and behavioural adaptation after an acute stress. For example, it has been reported that prenatally exposed rats exhibit impaired learning and coping strategy under stressful situations in a forced swim test (Welberg et al., 2001). Prenatally stressed rats have also been reported to demonstrate attenuated fear extinction in a contextual and cued fear conditioning test (Green et al., 2011b, Wilson et al., 2013, Markham et al., 2010).

Overall, the current evidence suggests that prenatal exposure to stress affects stress response in offspring, manifested as an increase in vulnerability and decrease in habituation to stressful stimuli.

3.1.2 Hypothesis and aim

I hypothesise that prenatal Dex exposure during the last week of gestation in rats may induce programming effects on offspring stress response system, potentially leading to hypersensitivity to stress in adult offspring. The aim of this study is to study the prenatal Dex effects on relevant aspects of a stress response including HPA axis activity, fear-related memory retention and behavioural adaptations.

The content of my current experiments include: a) measuring the basal and stress-induced HPA axis activity; b) evaluating unconditioned fear and anxiety-like behaviour on an elevated plus maze, open-field and contextual fear conditioning test; and c) evaluating fear conditioning and fear extinction in a contextual fear condition test.

3.2 Methods

3.2.1 Animals

Subjects were adult Wistar rats, bred from 36 adult pairs (Charles River, UK) at the University of Edinburgh. Animals were maintained and treated under conditions detailed in 2.2.

Female rats were housed individually and timed mating was conducted. Following vaginal plugging (denoted as E1) pregnant dams were randomly assigned to Dex or vehicle (Con) treatment. From E15-E21 inclusive, dams were given a daily subcutaneous injection of either Dex (100µg/kg body weight, dissolved in 4% ethanol, 0.9% saline) or an equivalent volume of vehicle (4% ethanol, 0.9% saline) (0.5ml/kg).

At birth (postnatal day 1), the offspring were weighed, sexed and litters culled back to eight per group, leaving 5 males and 3 females per litter where possible. Litters were then left undisturbed until weaning at 3 weeks of age, whereupon female offspring were culled and male pups were housed in same groups and left undisturbed until adulthood.

3.2.2 Plasma CORT levels

Animals (n= 9 per group) were assessed for basal plasma CORT levels in adulthood (aged 139.61 ± 1.33 S.D. days). Blood samples were obtained by making a small incision near the tip of the tail (Flutterm et al., 2000) at two time points, 07.00 and 19.00 h. Rats were directly taken from their home cage and sampled with minimal stress.

Another set of animals were used for assessment of stress-induced plasma CORT levels ten minutes after the elevated plus-maze test (n=CON:11, DEX:10, aged 72.0 ± 3.95 days). Animals were killed by rising concentration of carbon dioxide and then decapitation. Trunk blood was collected from the inferior vena cava. All blood samples were collected using EDTA coated capillary blood collection tubes (Microvette® CB-300) (Sarstedt) and centrifuged for

5 min at 13000 rpm at room temperature. The plasma was then transferred into a new Eppendorf™ tube and stored at -80°C . Plasma CORT concentrations were analysed by Dr Emad Al-Dujaili using enzyme-linked immunosorbent assay (ELISA)(Al-Dujaili et al., 2009).

3.2.3 Elevated plus-maze

Animals (n=CON:11, DEX:10) were aged 72.0 ± 3.95 S.D. days at testing. The elevated plus-maze was a cross-shaped platform positioned 80 cm above the floor with two open arms and two closed arms (70 cm x 12 cm x 17 cm) on opposing sides of a central area (10 cm x 10 cm). At the start of the test a rat was put in the central area facing one of the open arms and allowed to explore the maze freely for 5 minutes, during which its behaviour was recorded by a video camera mounted above the maze. Following each session the apparatus was wiped clean with 70% ethanol to remove any olfactory cues. An experimenter blind to treatment groups handled the animals and analysed the video recordings. The following parameters were considered: number of open arm entries, total number of arm entries and time spent on the open arms.

3.2.4 Open field

Animals (n= 12 per group) were aged 70.75 ± 4.46 S.D. days at testing. The open field apparatus was an illuminated black square box (90cm × 90cm × 60cm high). The field was divided into 9 equal segments. At the start of the test, a rat was placed at the edge of the box, in the middle of one of the surrounding walls, and allowed to explore the field freely for 5 minutes, during which its behaviour was recorded by a video camera mounted above the maze. Starting position was counterbalanced between groups. The field was cleaned by 70% ethanol to remove any possible olfactory cues between each animal. An experimenter blind to treatment groups handled the animals and analysed the video recordings. The parameters of interest were determined as: total number of grid crossings, number of entries to the central square and time spent in the central square.

3.2.5 Contextual fear conditioning

Animals (n=CON:12, DEX:16) were aged 74.21 ± 5.99 S.D. days at the start of testing. The contextual fear conditioning apparatus was a shock chamber (30 × 25 cm, 32 cm high, 16 shock bars, Coulbourn Instruments, PA, USA). The fear conditioning system was controlled via a shocker-scrambler unit automated by the FreezeFrame software (Coulbourn Instruments). The protocol consisted of a first day of training, a second day of retention (extinction training) and a third day of extinction. On the first day, rats were individually put into the chamber and allowed to explore the compartment for 2 min before receiving a 2s, 0.5 mA foot shock, after which they were left for a further 1 minute to allow for assessment of unconditioned freezing behaviour. Approximately 24 hours after the initial exposure, animals were returned into the same chamber for 10 min without receiving further foot shock to allow for assessment of fear consolidation and retrieval. After another 24 hours animals were again put back into the same chamber for another 10 min to allow for assessment of fear extinction. Animal behaviour was recorded at all times via a video camera mounted on the roof of the chamber. The apparatus was cleaned by 70% ethanol to remove any possible olfactory cues between each animal. Animals were coded by letters to blind treatment groups before the start of the experiment. An experimenter blind to treatment groups (Anna Moon, University of Cardiff) analysed the video recordings. Percentage time of freezing (defined as absence of all body movements except breathing) was examined for day 1 over the post-shock 1 min, and for day 2 and day 3 in blocks of 2 min over 10 min.

3.2.6 Statistical analysis

Data were analysed using generalised linear models (GLMs, JMP statistical software, SAS Institute, Cary, NC, USA). All data were checked for homogeneity of variance and normality of distribution. All statistical models investigated the main effect of Group. Litter (nested within group) was fitted as random variable to account for the use of more than one animal per litter. For contextual fear conditioning, GLMs were used to investigate the effect of Group on unconditioned freezing over the 1 min post-shock on day 1. For day 1 and day 2 data, GLMs were used to investigate the effect of Group (CON, DEX), Day (Day 1, Day 2) and all two-way interactions of these terms on percentage of time spent freezing. For day 2 and day 3 data, GLMs were used to investigate the effect of Group (CON, DEX), Day (Day 2, Day 3), Block (0-2 min, 2-4 min, 4-6 min, 6-8 min, 8-10 min), and all two-, three-way interactions of these terms on percentage of time spent freezing. Data were presented as mean value per group \pm S.E.M.

3.3 Results

3.3.1 Body weight

Prenatal Dex treatment significantly reduced birth weight in both male and female offspring, ($F_{1,14.97}=14.64$, $P=0.002$) ($n=60-83$ per sex/group). Female pups were significantly smaller than their male counterparts at birth, ($F_{1,253.6}=38.36$, $P<0.0001$). Analysis of body weight at weaning at three weeks of age ($n=27-44$ per sex/group) revealed a significant treatment effect in male pups ($P=0.020$), but not in female pups ($P=0.160$). Female pups were still lighter than male pups at weaning, ($F_{1,122.1}=19.69$, $P<0.0001$). The reduced body weight in prenatally Dex-treated male offspring persisted into adulthood, ($F_{1,15.8}=6.77$, $P=0.019$) ($n=42-44$ per group). (Figure 3.1)

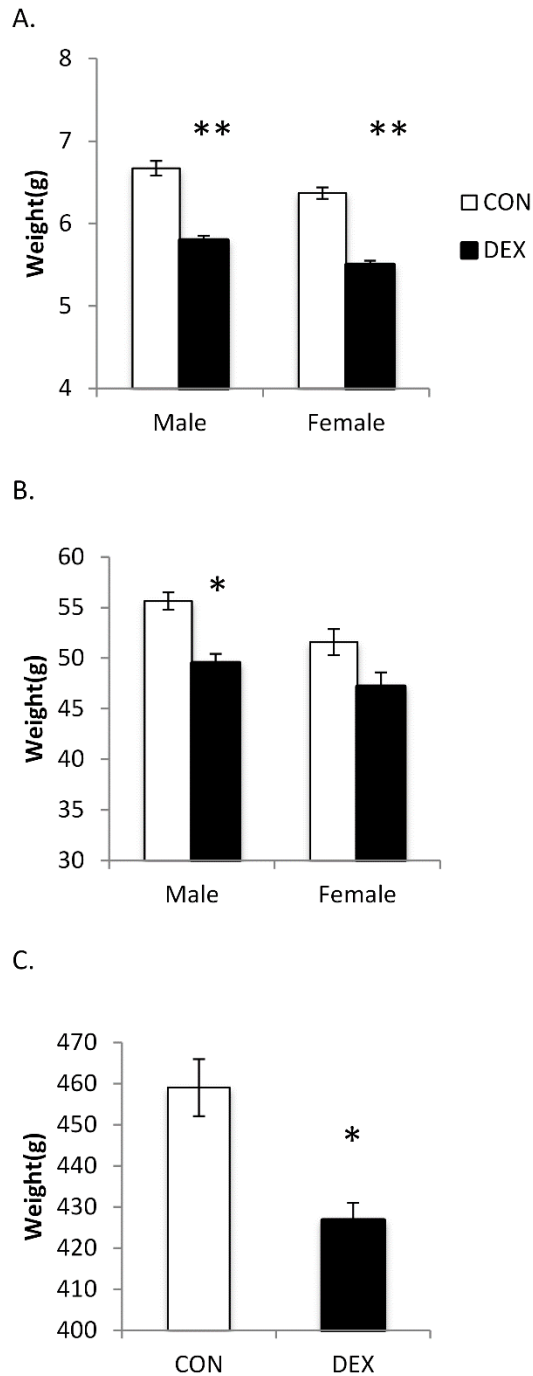


Figure 3.1 Effects of prenatal Dex exposure on offspring body weight

Prenatal Dex treatment significantly reduced birth weight in both male and female offspring (A); at weaning, the reduction in body weight induced by prenatal Dex exposure was only seen in male offspring (B); the reduced body weight in prenatally Dex-exposed male offspring persisted through adulthood (C). Data are presented as mean \pm S.E.M. * $P < 0.05$ versus control. ** $P < 0.01$ versus control.

3.3.2 Plasma CORT levels

Figure 3.2 shows that animals from both groups demonstrated a typical diurnal pattern of baseline CORT concentration changes. There were no significant differences between the two experimental groups in either the morning ($F_{1, 5.55}=0.12$, $P=0.738$) or evening basal plasma CORT levels ($F_{1, 8.34}=0.47$, $P=0.512$). Both groups of animals had similar elevation in plasma CORT concentrations after exposure to an acute elevated plus-maze stress, ($F_{1, 9.84}=0.40$, $P=0.543$).

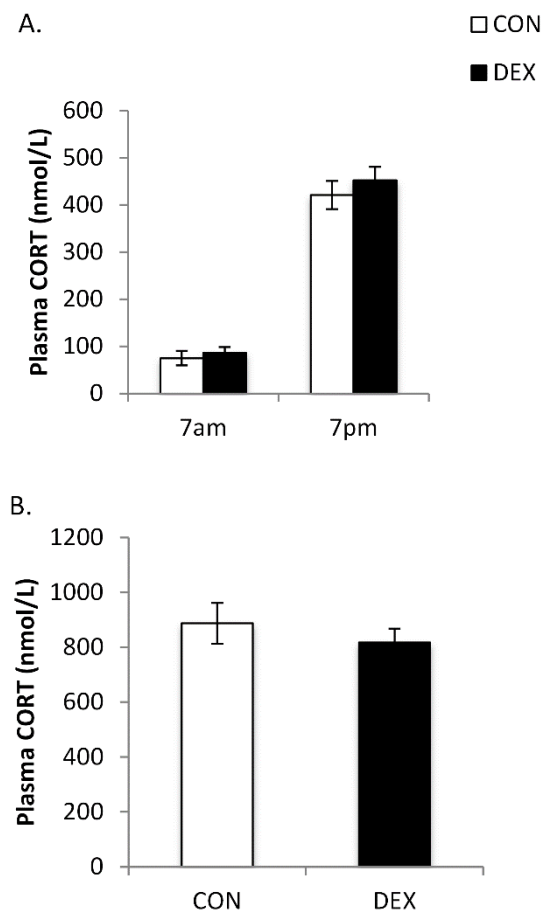


Figure 3.2 Effects of prenatal Dex exposure on basal and stress-induced plasma CORT levels in adult offspring

There were no significant differences between the two experimental groups in either basal CORT levels at 7am and 7pm (A), or stress-induced plasma CORT elevation 10 min following an acute stress (B). Data are presented as mean \pm S.E.M. $n=9-11$ /group.

3.3.3 Elevated plus-maze

Figure 3.3 illustrates the performance on an elevated plus-maze. Prenatal Dex exposure had no effect on number of entries to the open arms, ($F_{1, 8.21}=0.61$, $P=0.458$). Both groups of rats spent similar amounts of time on the open arms, ($F_{1, 9.81}=0.51$, $P=0.492$). There were no significant differences between the two groups in total number of arm entries, ($F_{1, 19}=2.41$, $P=0.137$).

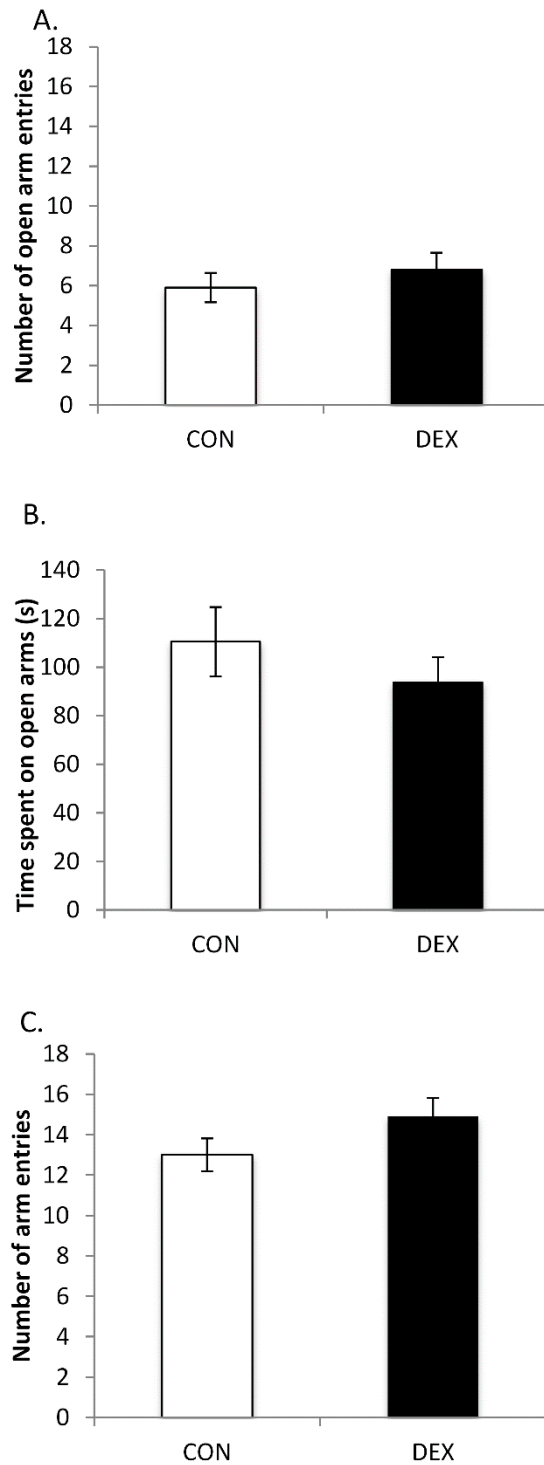


Figure 3.3 Performance on an elevated plus-maze

There were no significant differences between the two experimental groups on number of open arm entries (A), time spent on open arms (B) or number of total arm entries (C). Data are presented as mean \pm S.E.M. $n=10-11$ /group.

3.3.4 Open field

Figure 3.4 illustrates the performance over a 5 min open field test. There were no significant differences between the two experimental groups on time spent in the central square ($F_{1, 9.95}=1.25$, $P=0.290$) or number of entries to the central square ($F_{1, 7.93}=2.10$, $P=0.186$). Student t-test revealed a significant effect of Group on total grid crossings ($P=0.037$), such that Dex-programmed rats had a higher number of grid crossings. However if litter was fitted as a random variable by employing the GLM, there was only a trend towards a difference in total grid crossings between the two groups, ($F_{1, 8.02}=4.00$, $P=0.080$).

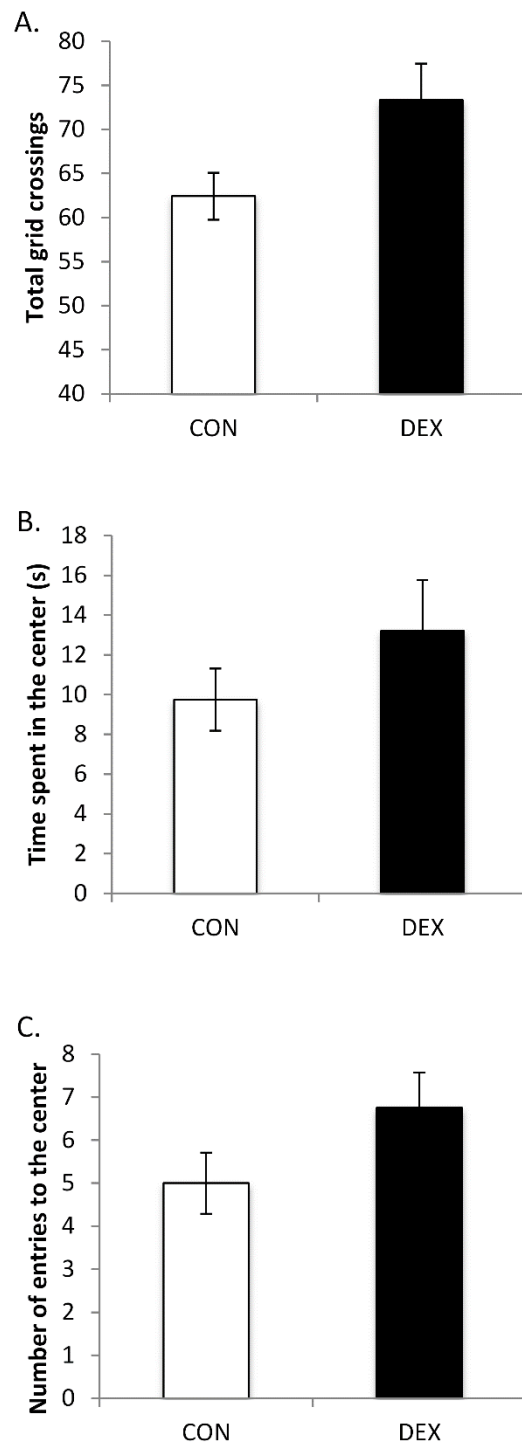


Figure 3.4 Performance on a 5 min open field test

Prenatal Dex exposure had no significant effect on total grid crossings (A), time spent in the central square (B) or number of entries to the central square (C). Data are presented as mean \pm S.E.M. n=12/group.

3.3.5 Contextual fear conditioning

Unconditioned freezing

There were no differences between the two groups in percentage of time spent freezing during the 1 min immediately after the food shock, ($F_{1,12}=0.29$, $P=0.600$). (Figure 3.5A)

Fear conditioning

Fear conditioning was assessed by comparing the percentage of time animals spent freezing during training (day 1) and retention (day 2). There was no main effect of Day ($F_{1,138}=0.77$, $P=0.381$) or Group ($F_{1,15.01}=0.39$, $P=0.543$), however there was a significant interaction between Group and Day, ($F_{1,138}=5.75$, $P=0.018$). Post Hoc LSMeans Student's *t* tests revealed that while control rats were able to retain high levels of conditioned freezing behaviour during retention, prenatally Dex-exposed rats had significantly reduced freezing behaviour during retention compared to training. (Figure 3.5B)

Fear extinction

Fear extinction was assessed by comparing the percentage of time animals spent freezing during retention (day 2) and extinction (day 3). There was a significant effect of Block, ($F_{4,234}=3.20$, $P=0.014$), such that less freezing behaviour was observed during the first block (0-2 min) compared to the next 2 blocks (2-6min). (Figure 3.5C) There was a main effect of Day ($F_{1,234}=25.82$, $P=0.0001$) and no main effect of Group ($F_{1,12}=0.94$, $P=0.351$), however there was a significant interaction between Group and Day, ($F_{1,234}=12.27$, $P=0.0005$). Post hoc LSMeans Student's *t* tests revealed a significant reduction in freezing behaviour during extinction in control animals but not in prenatally Dex-exposed animals. (Figure 3.5B)

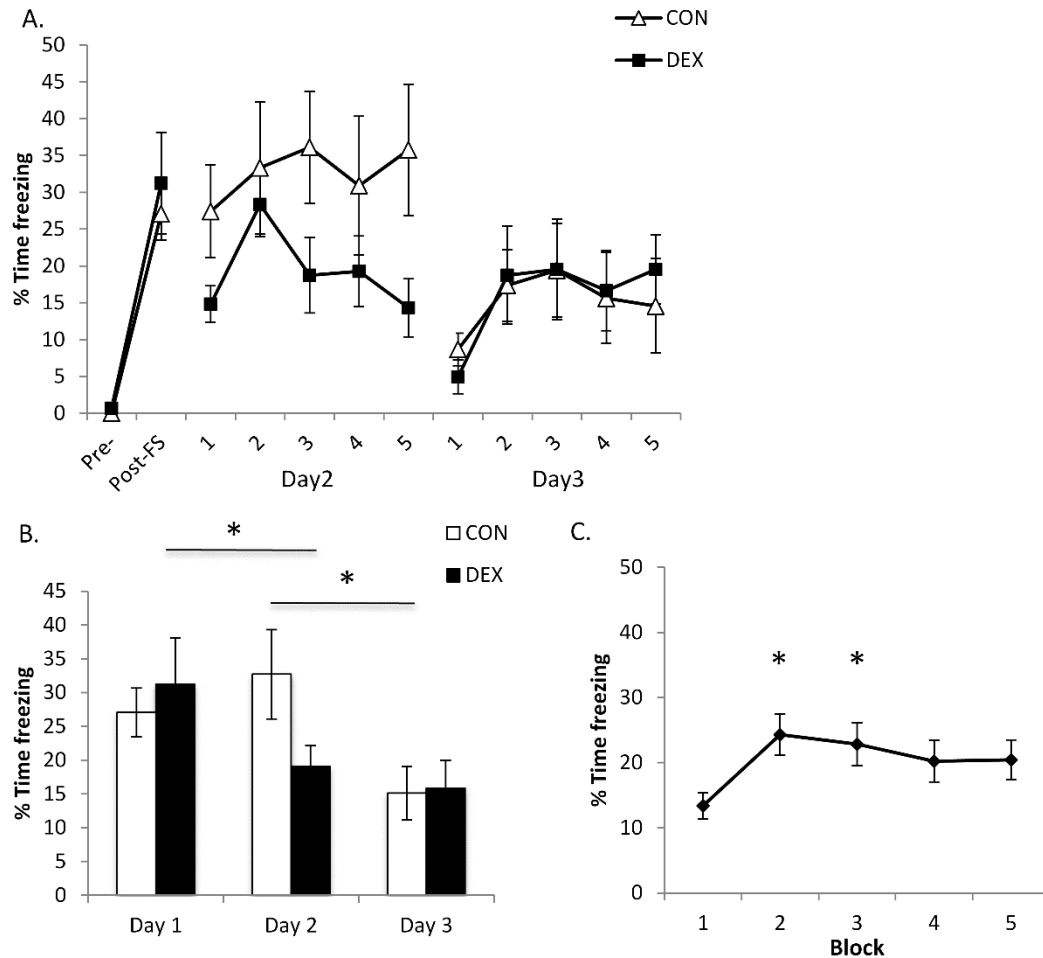


Figure 3.5 Performance on a contextual fear conditioning test

There were no significant differences between the two groups in unconditioned freezing (post-FS) (A); prenatal Dex treatment affected the time-course of freezing during retention and extinction, such that compared to controls prenatally Dex-exposed rats failed to develop conditioned freezing, and they again failed to demonstrate an elimination of freezing behaviour during extinction (B); there was a significant effect of Block during retention and extinction, such that all animals demonstrated significantly higher freezing behaviour about the time when they had first received the foot shock (at 2min) ($P=0.014$) (C). Data are presented as mean \pm S.E.M. $n=12-16/\text{group}$. * $P < 0.05$ versus block 1 (C).

3.4 Discussion

The experimental findings of my current study can be summarized as: a) prenatal exposure to Dex during the last week of gestation significantly reduced birth weight in offspring; b) prenatal Dex treatment had no effect on baseline or stress-induced plasma CORT levels in the adult offspring; c) there were no indications of group difference in unconditioned fear and anxiety-related behaviour as evaluated on an elevated plus-maze, open field and contextual fear conditioning test; d) prenatally Dex-exposed rats exhibited impaired fear conditioning during a contextual fear conditioning test and a trend towards increased spontaneous locomotor activity in an open field.

The current data confirmed that Dex treatment during the last week of gestation in rats reduces birth weight in offspring, in line with previous findings (Welberg et al., 2001, Nyirenda et al., 1998, Levitt et al., 1996). Increasing evidence suggests that low birth weight is associated with increased risks for neuropsychiatric disorders in later life, including autism (Pinto-Martin et al., 2011), schizophrenia (Abel et al., 2010) and ADHD (Breslau et al., 1996). Thus, this animal model may provide a potential tool to study related human conditions that are associated with low birth weight and to investigate the possible mechanisms underpin fetal programming of neuropsychiatric disorders.

Previous studies have reported a full ‘catch up’ in body weight in prenatally Dex-exposed rats at weaning (Welberg et al., 2001), however in my current study the reduced body weight in prenatally Dex-treated male offspring persisted until adulthood. The postnatal catch-up growth could be an additional risk for later diseases (Bavdekar et al., 1999, Leon et al., 1996, Law et al., 2002, Eriksson et al., 1999), however it is not in itself a necessity for programming effects to occur, as persistent behavioural and molecular changes were still evident even without a normalization of body weight in adult rats (Welberg et al., 2001).

There were no indications of HPA axis programming from my current experiment, as all animals exhibited similar levels of basal and stress-induced CORT concentrations. This result appears to contrast with some previous studies, in which researchers reported increased basal CORT levels (Green et al., 2011b) or heightened CORT secretion 30 min after an acute stress exposure in offspring of stressed dams (Wilson et al., 2013). Differences in animal strains (Sprague Dawley rats in these two studies) and prenatal treatment (restraint stress in the Green study and repeated variable stress in the Wilson study) may partly account for the variable results. In fact, experimental findings vary in this field, with some report HPA hypersensitivity only in juvenile prenatally stressed rats, which normalizes in adulthood (Takahashi et al., 1992, Takahashi and Kalin, 1991), while others report sex-specific programming of the HPA axis where the effects are only seen in the female offspring (Weinstock et al., 1992, Szuran et al., 2000).

There was no evidence of prenatal Dex programming on unconditioned fear and anxiety-related behaviour from my current study, as evaluated by a) avoidance of anxiogenic locations on an elevated plus-maze and open field; b) exploratory behaviour in an open field; and c) acute freezing behaviour on a contextual fear conditioning test.

Aversion to the open arms, including reduced entries and less time spent on the open arms, has been validated to be specific indicators for an anxious phenotype (Pellow et al., 1985). In my current experiment, I found no significant differences between the groups in tendencies to avert open arms on an elevated plus-maze. Although contrast to a number of previous studies (detailed in 3.1), similar negative findings have been reported by another group (Wilson et al., 2013). Large variations in animal strain, prenatal stress regimen and offspring age may all contribute to a portion of the controversial results. Notably, in one previous study that used a same animal model, researchers reported a trend towards lower entries to the open arms in

prenatally Dex-treated rats ($P=0.05$, $n=8-10$ per group), with no significant differences in amount of time spent on open arms (Welberg et al., 2001). There was also a significant reduction of total number of arm entries in prenatally Dex-treated rats ($P<0.05$), which might have confounded the analysis of number of open arm entries. In addition, possible differences in rearing conditions and the use of different experimental apparatus and the settings (these experiments were performed in different animal facilities) may also contribute to the discrepancies. For example, there is evidence that environmental noise can confound prenatal Dex effects in Wistar rats (O'Regan et al., 2010). To clarify these possibilities, I have replicated the current results with two other cohorts of rats in the same and different animal facilities. Despite variations in rearing environment and experimental settings, my results from the three cohorts were consistent ($n=\text{CON}:36$, $\text{DEX}:39$ in total, data not shown) with all three replications showing a lack of prenatal Dex programming effects on unconditioned elevated plus-maze behaviour.

Similar to the open arms on an elevated plus-maze, the centre of an open field represents an anxiogenic location, and it has been suggested that the amount of time spent in the centre and entries to the centre of an open field are also indicators for anxiety in rats (Vallee et al., 1997). My current data on these parallel measurements supported the elevated plus-maze results, suggesting no differences in avoidance of anxiogenic locations between the two experimental groups.

Reduced exploratory behaviour is another aspect of fear and anxiety-related behaviour, which can be assessed by ambulation (total grid crossings) in an open field test. However this measurement can sometimes encompass functionally unrelated activities such as locomotion (Prut and Belzung, 2003), especially when antagonistic actions of reduced exploration (Welberg et al., 2001) and increased spontaneous locomotion (Diaz et al., 1997, Wilson et al.,

2013) have both been reported following prenatally stress exposure. In some studies, horizontal open field activities such as grid crossings are used as indicators of locomotion, whereas vertical activities such as rearing and sniffing are used as indicators of exploration (Wilson et al., 2013). In other studies, the overall open field behaviour is analysed as spontaneous locomotor activity, researchers have however distinguished the first 10 min of open field as exploratory period and the next 20 min as habituation period (Diaz et al., 1997). In my current experiment there was a tendency towards increased total grid crossings over the 5 min open field test in prenatally Dex-programmed rats. The current finding is consistent with a good number of studies in which offspring of stressed dams exhibit increased locomotor activities, especially during the initial 5 min of exposure to the open field (Wilson et al., 2013, Vallee et al., 1997, Gue et al., 2004, Markham et al., 2010). In particular, prenatal exposure to glucocorticoids has been shown to induce increased spontaneous locomotion in rats (Diaz et al., 1997). This is potentially interesting because hyperactivity is a common symptom for neuropsychiatric disorders such as ADHD (Grizenko et al., 2008), and one risk of which has been associated with prenatal stress.

Fear and anxiety-related behaviour can also be measured by acute unconditioned freezing behaviour in a contextual fear conditioning test. Again, there were no differences between the groups on percentage of time spent freezing over the 1 min immediately after receiving the electrical foot shock. Taken together, the above results suggest there were no effects of prenatal Dex exposure on unconditioned fear and anxiety-related behaviour in the adult offspring in rats.

In addition, the contextual fear conditioning test also allows for assessment of hippocampus-dependent acquisition, consolidation, retrieval and extinction of fear-related memory (Rudy et al., 2004). Contextual fear conditioning assesses for the ability to associate fear with a related

context and to consolidate and retrieve this memory upon re-exposure. Fear extinction assesses for the ability to reconsolidate a previously learned memory and adapt to the new experience in which the context is no longer predictive of aversive electric foot shock. Interestingly, in the current contextual fear conditioning test, prenatally Dex-exposed rats demonstrated altered time-course of freezing behaviour during both retention and extinction. Compared to controls, prenatally Dex-exposed rats failed to develop conditioned freezing behaviour during retention, and as a consequence, they also failed to eliminate the freezing behaviour during extinction. Collectively, my current study suggests that prenatal Dex treatment resulted in impaired hippocampus-dependent contextual fear recall 24 hours after the initial conditioning in adult male offspring. This result is contrary to previous reports, in which researches found no effects of prenatal exposure to stress on percentage of time spent freezing during a contextual fear recall 24 hours (Markham et al., 2010) and 48 hours (Wilson et al., 2013) after the initial conditioning. Discrepancies between studies could be due to variations in animal strains (Sprague Dawley rat in these two studies), prenatal stress regimens (repeated variable prenatal stress from E14 in these two studies). There are also considerable differences in fear conditioning protocols, for example, the Markham study used four tone-shock pairings (1.0mA, lasting 1s, 90s apart), the Wilson study used two foot shocks (0.7mA, lasting 1000ms, 1 min apart), and both studies measured fear retention only for a period of 5 min.

The current data on fear extinction is inconclusive because prenatally Dex-exposed rats failed to develop an adequate conditioning in the first place. Extension of conditioning training in prenatally Dex-treated animals would enable reliable fear conditioning to investigate any possible effect of prenatal Dex treatment on fear extinction. This is potentially interesting because previous studies report prenatal exposure to stress leads to impaired contextual (Wilson et al., 2013) or cued fear extinction in male rat offspring (Green et al., 2011b, Markham et al., 2010).

Taken together, the results from my current experiment suggest that prenatal exposure to Dex during the last week of gestation in rats had no observable effect on acute stress responses, but resulted in impaired hippocampus-related contextual fear conditioning in the adult offspring. The current experiment also revealed a trend indicating increased spontaneous locomotor activity in prenatally Dex-exposed adult rats.

**Chapter 4 Glucocorticoid programming:
effects on cognition in adult rats**

4.1 Introduction

4.1.1 Introduction

Several human studies have provided evidence that maternal anxiety and stress during pregnancy affect cognitive development of the child, including intellectual, language, impulsion and memory abilities (Laplante et al., 2004, Buitelaar et al., 2003, Van den Bergh et al., 2005, Buss et al., 2011). In laboratory animals, prenatal exposure to stress has been associated with impaired performance on cognitive tasks related to hippocampus and PFC function, including contextual or cued fear conditioning and extinction (Markham et al., 2010, Green et al., 2011b, Wilson et al., 2013), spatial reference memory (Lemaire et al., 2000, Son et al., 2006, Yang et al., 2006, Brabham et al., 2000, Markham et al., 2010) and working memory (Markham et al., 2010, Gue et al., 2004).

In the previous chapter, I have demonstrated that prenatal exposure to Dex induced impaired hippocampus-related contextual fear conditioning in the adult offspring in rats. I thus wanted to investigate further on other aspects of cognitive outcomes induced by prenatal Dex treatment, in particular PFC-related cognitive function. This is interesting firstly because PFC dysfunctions are key characteristics of a variety of neuropsychiatric disorders such as schizophrenia, depression and anxiety disorders (Murphy et al., 1999, Goldman-Rakic, 1994, Kuperberg and Heckers, 2000). Secondly, there is evidence that maternal anxiety during pregnancy leads to PFC-related cognitive impairments in adolescents (Mennes et al., 2006, Van den Bergh et al., 2005).

In this chapter, I aimed to further investigate the impact of prenatal Dex exposure on cognition in adult rat offspring by employing a range of behavioural measures, including a) a spatial reference memory task with reversal learning; b) a delayed matching to position (DMTP) task; and c) an intra-dimensional/extra-dimensional (ID/ED) attentional set-shifting test.

The spatial reference memory task has been developed and validated as a robust measure of hippocampus-dependent spatial navigation and reference memory (D'Hooge and De Deyn, 2001). The reversal-learning phase requires the animal to withhold a formerly learned response and adapt its behaviour according to changes in stimulus–reward contingencies. The neural substrate underlying the reversal learning has been ascribed to the orbital PFC (McAlonan and Brown, 2003).

The DMTP task allows for assessment of working memory and behavioural flexibility, both of which have been ascribed to the PFC (Kesner, 2000). In humans, working memory involves short-term retention of visuo-spatial information and executive function of manipulating this information to guide for subsequent responses (Baddeley, 1992). The classic DMTP task is carried out in a radial arm maze, where working memory deficits can be characterized by either the ‘delay’ component (delay-dependent or delay independent deficits) (Dunnett et al., 1989), or the ‘spatial matching’ component (working memory correct and working memory incorrect errors) (Bimonte et al., 2000). In the current DMTP task, which was conducted in a Morris water maze, only the ‘delay’ part allows for assessment of working memory, whilst the ‘spatial matching’ component has been adapted and is more indicative of response inhibition and behavioral flexibility. Lesion studies in rats suggest that an efficient performance on the DMTP task depends on a coordinated interaction between the hippocampus and PFC (Floresco et al., 1997, Seamans et al., 1995).

The ID/ED attentional set-shifting test allows for assessment of attentional shifts between different perceptual dimensions. The behavioural flexibility assessed on the above two tasks are intra-dimensional shifts, in which the behavioural shifts are made within one particular dimension. The ID/ED task assesses not only intra-dimensional shifts (within one perceptual

dimension), but also extra-dimensional shifts (between perceptual dimensions). My current experiment followed a standard protocol developed by researchers from the University of St. Andrews (Birrell and Brown, 2000). It is an analogue to the visual discrimination task, which has been decomposed from the original human Wisconsin Card Sorting Test that assesses for frontal lobe function (Roberts et al., 1992). The neurological substance that underlying the extra-dimensional attentional shifts has been ascribed to medial PFC in rats (Birrell and Brown, 2000).

4.1.2 Hypothesis and aim

I hypothesise that overexposure to glucocorticoids during the last week of gestation may affect cognitive function in adult Wistar rat offspring. The aim of this study is to provide a more comprehensive picture of the long-term impact of prenatal Dex exposure on offspring cognition, particularly those associated with hippocampus and PFC functioning.

The behavioural tasks involved in this chapter include: a) a spatial reference memory task with reversal learning; b) a delayed matching to position (DMTP) task; and c) an intra-dimensional/extra-dimensional (ID/ED) attentional set-shifting test.

4.2 Methods

4.2.1 Animals

Subjects were adult Wistar rats, bred from 20 adult pairs (Charles River, UK) at the University of Edinburgh. Animals were maintained and treated under conditions detailed in 2.2.

Female rats were housed individually and timed mating was conducted. Following vaginal plugging (denoted as E1) pregnant dams were randomly assigned to Dex or vehicle (Con) treatment. From E15-E21 inclusive, dams were given a daily subcutaneous injection of either Dex (100µg/kg body weight, dissolved in 4% ethanol, 0.9% saline) or an equivalent volume of vehicle (4% ethanol, 0.9% saline) (0.5ml/kg).

At birth (postnatal day 1), the offspring were weighed, sexed and litters culled back to eight per group, leaving 5 males and 3 females per litter where possible. Litters were then left undisturbed until weaning at 3 weeks of age, whereupon female offspring were culled and male pups were housed in same groups and left undisturbed until adulthood.

4.2.2 Spatial reference memory with reversal learning

Animals (n=12 per group) were assessed on the spatial reference memory with reversal learning task at age 100.38 ± 6.39 S.D. days at the start of testing. This task was carried out in an open-field water maze. The maze was a water tank of 2m in diameter and 0.5m in depth, filled with water made opaque by mixing it with 300ml of liquid latex (temperature $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$). An escape platform (12 cm in diameter) was submerged 1.5 cm below the water surface. The maze was surrounded by a collection of prominent two- and three-dimensional visual cues. Animal behaviour was monitored using a video tracking system via a camera fixed above the centre of the maze, and was analysed by a water maze software (Tracker- P. Spooner, Edinburgh, UK). For each trial, the latency to reach the platform, path length, and swimming speed was measured. Four points of equal spacing along the circumference of the pool were signified as starting points: North (N), East (E), South (S) and West (W). Likewise, the swimming pool was conceptually divided into four quadrants accordingly, NE, SE, SW and NW.

The spatial reference memory with reversal learning task consisted five days of initial training and another five days of reversal learning. For each rat, the platform location remained constant (either NE or SW quadrant, 60 cm from the wall, counterbalanced between groups) throughout the first five days of training, and was moved to the opposite quadrant (SW or NE) for the following 5 days of reversal learning. On each day, a rat was given five trials in a row, with an inter-trial interval (ITI) of approximately 15s. At the start of each trial the rat was carefully lowered into the water, facing one of the four starting points. Starting position was counterbalanced between groups. The sequence of the starting positions was randomized, with at least one start at N, E, S and W on each day, avoiding repeats for two consecutive trials.

The first trial of each day was run as a probe trial. Probe trials used the “Atlantis” platform (Spooner et al., 1994), which was pushed down at first to render it unavailable and set to rise at 60s by using a computer-controlled electromagnet, and the animal was given a further 30s to find it. The percentage of time spent near the two possible platform locations (radius 20.0) during the first 60s was analysed. The following trials on each day (trials 2-5) were standard trials, during which rats were allowed 120 seconds to find the platform before being ushered towards it. Once a rat reached the platform, it was allowed to remain on the platform for 30s before being taken off and moved on to the next trial.

4.2.3 Delayed matching to position

Animals were assessed on the DMTP task at age 90.92 ± 12.11 S.D. days at the start of testing. The DMTP task was carried out in the same water maze apparatus as detailed in 4.2.2. This task comprised seven days of initial training followed by three blocks of delayed tests, each of these blocks continued for five days. On each day, the platform location was different, randomly selected from one of the 28 possible locations situated in an inner (1m in diameter) and outer (1.5m in diameter) ring of the swimming pool (Figure 4.1). For a given rat, the platform location remained the same for all the trials on that day. Each rat received four trials per day, the starting position for each trial was considered in relevance to the location of the platform, designated as far right (FR), far left (FL), near right (NR) and near left (NL). On each day, the sequence of starting positions was randomized for the four trials with at least one start at each. The sequences of the daily platform locations and relative starting positions were carefully designed to counterbalance between and within groups to avoid any clear recognizable pattern.

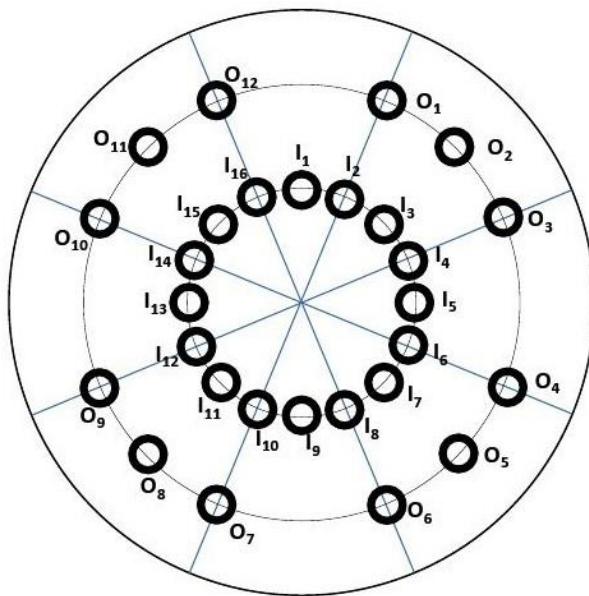


Figure 4.1 Platform locations in a DMTP task

DMTP- training phase

During the training phase all rats were given four consecutive trials per day for seven days. The platform was placed according to the pre-designed location, and submerged 1.5cm below the water surface before the trials began. On each trial the rat was allowed 120s to find the platform, before being guided towards it. Once the rat climbed on the platform it had 30s to remain on the platform. An experimenter would then remove the rat and allow a 15s ITI before moving on to the next trial.

DMTP- delay phase

During the delay phase, the training protocol was the same as before, except that we introduced a delay time of 15 seconds, 20 minutes, or 2 hours between the first and the second trial, during which time animals were dried and returned to their home cage. The delay phase included three blocks, each continued for five days, with two days of break in between. Within each block, rats were assigned to one of the three delays in a counterbalanced fashion, and remained on that delay for five days. On the last day of each block, the second trial was run as a probe, to allow for assessment of working memory. Probe trials used the same “Atlantis” platform, as detailed in 4.2.2, during which the percentage of time spent near the most recently trained platform location (radius 20.0) was analysed.

4.2.4 ID/ED attentional set-shifting

Animals (n=CON:10, DEX:12) were assessed using the ID/ED test at age 144.00 days \pm 10.11 S.D. days at the start of the test. The apparatus was adapted from a home cage. A fixed plastic panel was set to divide one third of the length of the cage into two sections. In each section, a ceramic bowl was placed in the middle, which can be filled with digging medium and scented with odour. Two removable dividers were set in between the waiting area and the two sections, which can be operated by the experimenter to allow or block the rat's access to each section.

ID/ED habituation:

Rats were maintained on restricted diet with free access to water during the whole experiment. On the night before the experiment, each rat was given a sawdust-filled ceramic bowl with 6 Honey Nut Loops (Kellogg, Manchester, UK) buried in it. On the next day, each rat was individually placed in the waiting area of the apparatus and allowed free access to both separated sections. A sawdust-filled digging bowl with one-half of a honey loop buried in it was placed in the middle of each section. Both bowls were rebated every 5 min, or after the rat had retrieved food from both sides. Baits were buried each time deeper until near the bottom. This process was repeated until the rat could reliably dig to retrieve rewards from both sides. After this, the rat was exposed to the six pairs of exemplars that would be used during the test (3 digging medium pair and 3 odour pair). For each rat, the six pairs of exemplars to be used were randomly chosen from a list of fourteen pairings (Table 4.1). Rats were trained to dig in any of these exemplars to retrieve treats before carrying on to the next stage. This might take up to several days, depending on the individual rat.

ID/ED training:

Before starting, each rat was accustomed to all the six pairs of exemplars again for another time. Afterwards, it was trained on two simple discrimination (SD) tests (all rats were trained

on the same training exemplars, which were not used again during testing). A SD test began by lifting up the dividers and exposing the rat to the two separated sections. Digging bowls were placed in the middle of each section, and were filled with the training pair of digging medium or odour-scented sawdust, depending on the perceptual dimension of the SD test (digging medium versus odour). On each trial, the correct exemplar remained the same, and only the bowl with the correct exemplar was baited with treat. The rat was allowed to explore both bowls on the first four trials to learn the correct digging medium or odour. On subsequent trials, if the rat started to dig in the wrong bowl, the trial was terminated and an error was recorded. The trials continued until the rat had reached a criteria of six consecutive correct trials.

ID/ED testing:

One day after training, rats were tested on a series of seven discriminations (Table 4.2). A SD test was the same as described in the training and involved only one perceptual dimension (for example, digging medium). For the compound discrimination (CD), a second dimension (odour) was added, but the correct and incorrect exemplar was the same as the SD. For intra-dimensional discrimination (ID), two new pairs of exemplars were introduced, but the relevant perceptual dimension was the same as the CD (digging medium). For extra-dimensional discrimination (ED), another two new pairs of exemplars were used and the relevant dimension was also changed (odour). For each reversal after the CD, ID and ED (Rev1, Rev2 and Rev3), the exemplars being used and the relevant dimensions remained the same, but the correct and incorrect exemplars were reversed. The criteria were the same as in the training, and the number of trials to reach six consecutive correct trials was recorded for each discrimination.

	Odour		Medium	
Training pairing	Oregano	Mint	Polystyrene	Confetti
Pairing 1	Ginger	Cinnamon	Coarse Hay	Small Hay
Pairing 2	Paprika	Sage	Sand	Grit
Pairing 3	Cloves	Turmeric	Coarse wood	Fine wood
Pairing 4	Coriander	Dill	Cigarette filters	Wool
Pairing 5	Tarragon	Fenugreek	Paper	Cotton wool
Pairing 6	Marjoram	Cumin	Coarse tea	Fine tea
Pairing 7	Carraway seeds	Thyme	Hama Beads	Baken Beans

Table 4.1 Stimulus exemplars

	Relevant Dimension	Irrelevant Dimension
Discriminations	Exemplars	Exemplars
SD	O1/O2	Non-dissociable
CD	O1/O2	M1/M2
CD Rev	O2/O1	M1/M2
ID	O3/O4	M3/M4
ID Rev	O4/O3	M3/M4
ED	M5/M6	O5/O6
ED Rev	M6/M5	O5/O6

Table 4.2 Sequence of discriminations in an ID/ED task

4.2.5 Statistical analysis

Data were analysed using GLMs (JMP statistical software, SAS Institute, Cary, NC, USA), followed by post hoc LSMeans Student's *t* tests, where appropriate. All data were checked for homogeneity of variance and normality of distribution. For all statistical model, animal identity (nested within litter and group) and litter (nested within group) were fitted as random variables to account for repeated measurements and the use of more than one animal per litter.

For the spatial reference memory with reversal learning task, GLMs were used to investigate the effects of Group, Trial, Day, and all two-, three-way interactions of these terms on latency to platform, path length, percentage of time near the wall and average swimming speed for all the standard trials (trials 2-5). For probe trials (trial 1), GLMs were used to investigate the effects of Group, Day and two-way interactions of these terms on percentage of time near the initial training platform.

For the DMTP task, data were analysed separately. For the training phase, GLMs were used to investigate the effect of Group, Trial, Day, and all two-, three-way interactions of these terms on latency to platform, path length, percentage of time near the wall and average swimming speed for all the trials (trial 1-4). For the delay phase, GLMs were used to investigate the effect of Group, Delay, Trial, and all two-, three-way interactions of these terms on latency to platform, path length, percentage of time near the wall and average swimming speed on all the trials from the first four days of each block. GLMs were also employed to investigate the effect of Group, Delay and two-way interactions of these terms on saved latency time from trial 2 to trial 1 on the same datasheet. For probe trials (the second trial on the last day of each block), GLMs were used to investigate the effect of Group, Delay, and two-way interactions of these terms on percentage of time spent near the most recently trained platform

location divided by the total amount of time spent near the other seven related locations, sitting at the vertices of an octagon.

For the ID/ED task, GLMs were used to investigate the effect of Group, Discrimination (SD, CD, CD Rev, ID, ID Rev, ED, ED Rev) and two-way interactions of these terms on number of trials to criteria. Reversal learning was assessed by animal performance on reversals (CD Rev, ID Rev, ED Rev) versus initial acquisitions (SD, CD, ID, ED). GLMs were used to investigate the effect of Group, Phase (initial, reversal) and two-way interactions of these terms on number of trials to criteria. For the ID and ED data only, GLMs were used to investigate the effect of Group, Discrimination (ID, ED), Sequence (odour to medium, medium to odour), and all two-, three-way interactions of these terms on number of trials to criteria. Data were presented as mean value per group \pm S.E.M.

4.3 Results

4.3.1 Spatial reference memory with reversal learning

Latency to platform

Figure 4.2 demonstrates the latency to platform over ten days of the spatial reference memory with reversal learning task. There was a significant effect of Day, ($F_{9, 917}=62.27$, $P<0.0001$). There was no main effect of Group ($F_{1, 3.676}=1.60$, $P=0.281$), however there was a significant interaction between Group and Day, ($F_{9, 917}=2.40$, $P=0.011$). Post hoc LSMeans Student's *t* tests revealed that prenatally Dex-exposed animals were significantly slower than controls in finding the platform on the first day of training ($P<0.05$). In addition, on the first day of reversal, there was a tendency that control animals took a longer time to learn the new platform location ($P=0.05$ by Student's *t*-test).

Probe trials

Figure 4.3 demonstrates the percentage of time spent near the initial training platform (radius 20.0) on probe trials. There was a significant effect of Day, ($F_{9, 189}=11.35$, $P<0.0001$), such that all animals spent increasingly more time around the training platform as days progressed, reaching a stable level by day 5. On day 7, after the reversal, the time they spent near the initial training platform dropped to a baseline level, which was not different from that seen on the first day of training. Both groups of animals performed equally on probe trials, as there was no main effect of Group ($F_{1, 4.722}=0.03$, $P=0.863$) and no interaction between Group and Day ($F_{9, 189}=0.72$, $P=0.690$).

Locomotor activity

There were no significant differences between the groups in average swimming speed during the whole experimental period, ($F_{1, 4.50}=0.71$, $F=0.443$). Likewise, no significant difference was found in percentage of time spent near the wall, ($F_{1, 6.77}=0.44$, $P=0.530$).

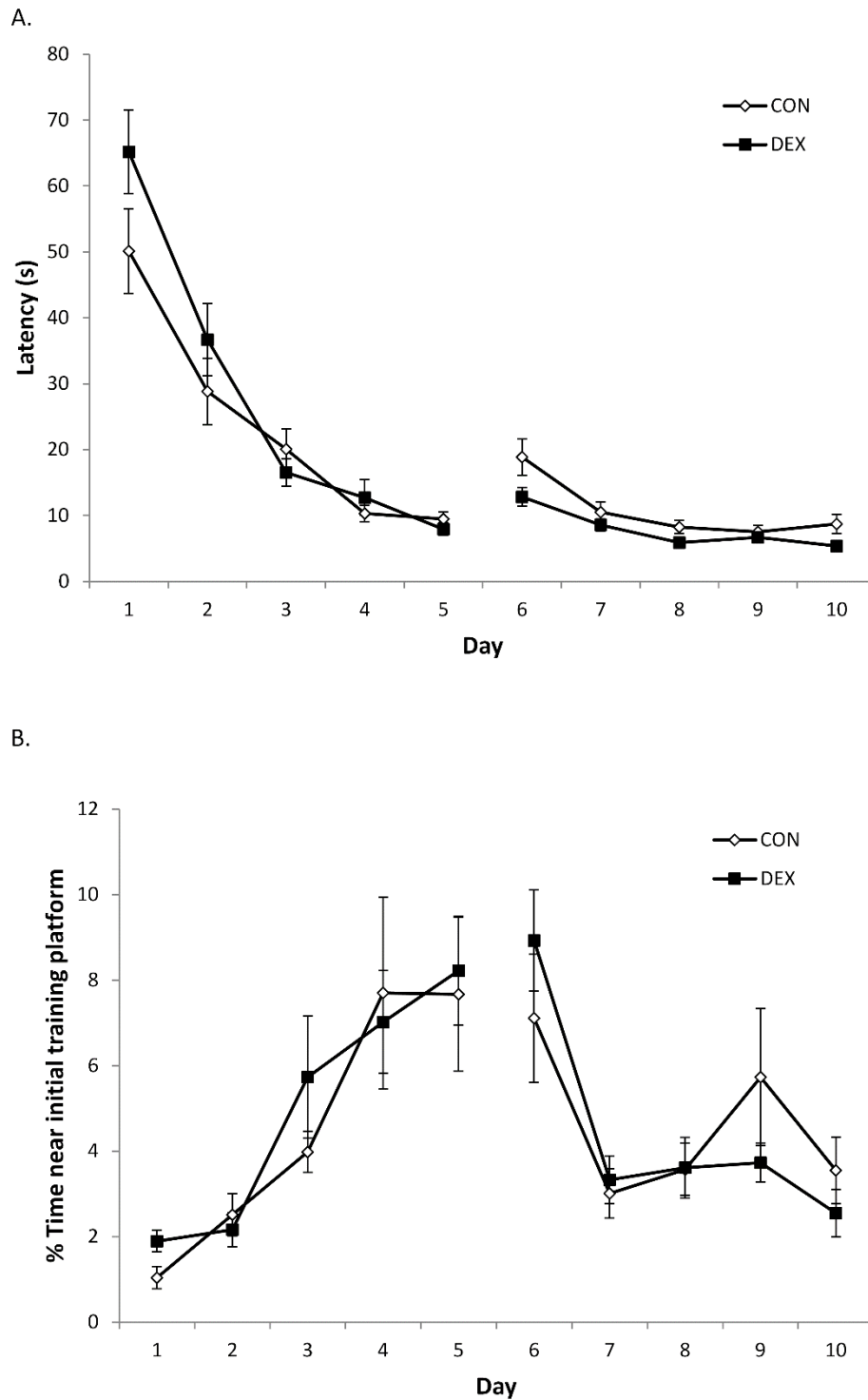


Figure 4.2 Performance on a spatial reference memory with reversal learning

There was a significant interaction between Group and Day in latency time to platform ($P=0.011$) (A); both groups of animals showed similar preference for the training platform location when memory was probed 24 hours after the last trial (B). Data are presented as mean \pm S.E.M. $n=12$ /group.

4.3.2 Delayed matching to position

Latency to platform in the DMTP training phase

Figure 4.3 illustrates the latency to platform during the training phase. Statistical analysis revealed a significant effect of Day ($F_{6, 594}=31.86$, $P<0.0001$), such that all animals improved their performance as days progressed, reaching a steady level by day 4. There was also a significant effect of Trial ($F_{3, 594}=31.35$, $P<0.0001$), as animals took significantly longer time to find the platform on the first trial compared to the other three within a day. Moreover, there was a significant effect of Group ($F_{1, 7.69}=8.16$, $P=0.022$), with Dex animals taking significantly less time than controls to find the platform (Figure 4.3B). There were no interactions between Group and Day, ($F_{6, 594}=0.68$, $P=0.666$). An identical pattern of performance was seen when path length was used as the dependent variable. (Data not shown)

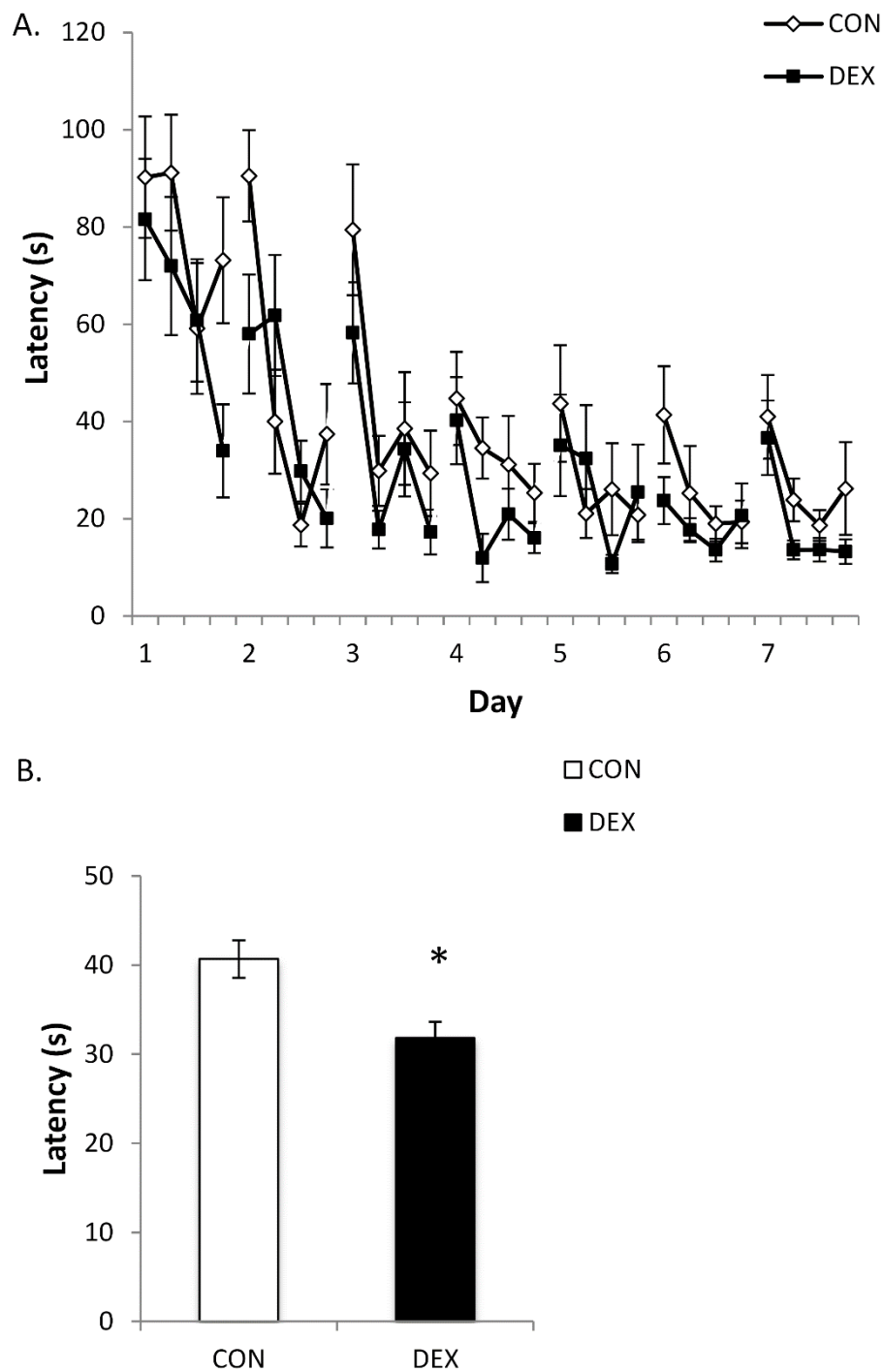


Figure 4.3 Performance during the training phase of a DMTP task

There was a significant effect of Group ($P=0.022$), with Dex animals taking significantly less time than controls to find the platform (B). Data are presented as mean \pm S.E.M. $n=12/\text{group}$. * $P < 0.05$ versus control.

Latency to platform in the DMTP delay phase

There was a significant effect of Trial, ($F_{3, 1034}=36.75$, $P<0.0001$). Post hoc analysis showed that animals had a shorter latency on the second trial compared to the first, with further improvements on the last trial. There was no main effect of Delay ($F_{2, 1034}=0.36$, $P=0.70$), however there was a significant interaction between Delay and Trial, ($F_{6, 1034}=2.38$, $P=0.028$). Post hoc LSMeans Student's t tests showed that animals were significantly faster on the second trial in the 15s and 2 h delay, whereas in the 20 min delay, animals only showed a significant improvement on the fourth trial compared to the first one. There were no three way-interactions between Group, Delay and Trial, ($F_{6, 1034}=1.39$, $P=0.217$). Notably, there was a significant effect of Group ($F_{1, 9,592}=5.58$, $P=0.041$), with Dex animals taking significantly less time to find the platform than controls. (Figure 4.4A-D)

Saved time from trial 2 to trial 1 in the DMTP delay phase

There was no significant effect of Group in saved time from the second trial to the first during the delay phase, ($F_{1, 8,371}=0.33$, $P=0.58$). There was no significant effect of Delay ($F_{1, 258}=0.76$, $P=0.38$) and no interaction between Group and Delay, ($F_{1, 258}=1.53$, $P=0.217$). (Figure 4.4E)

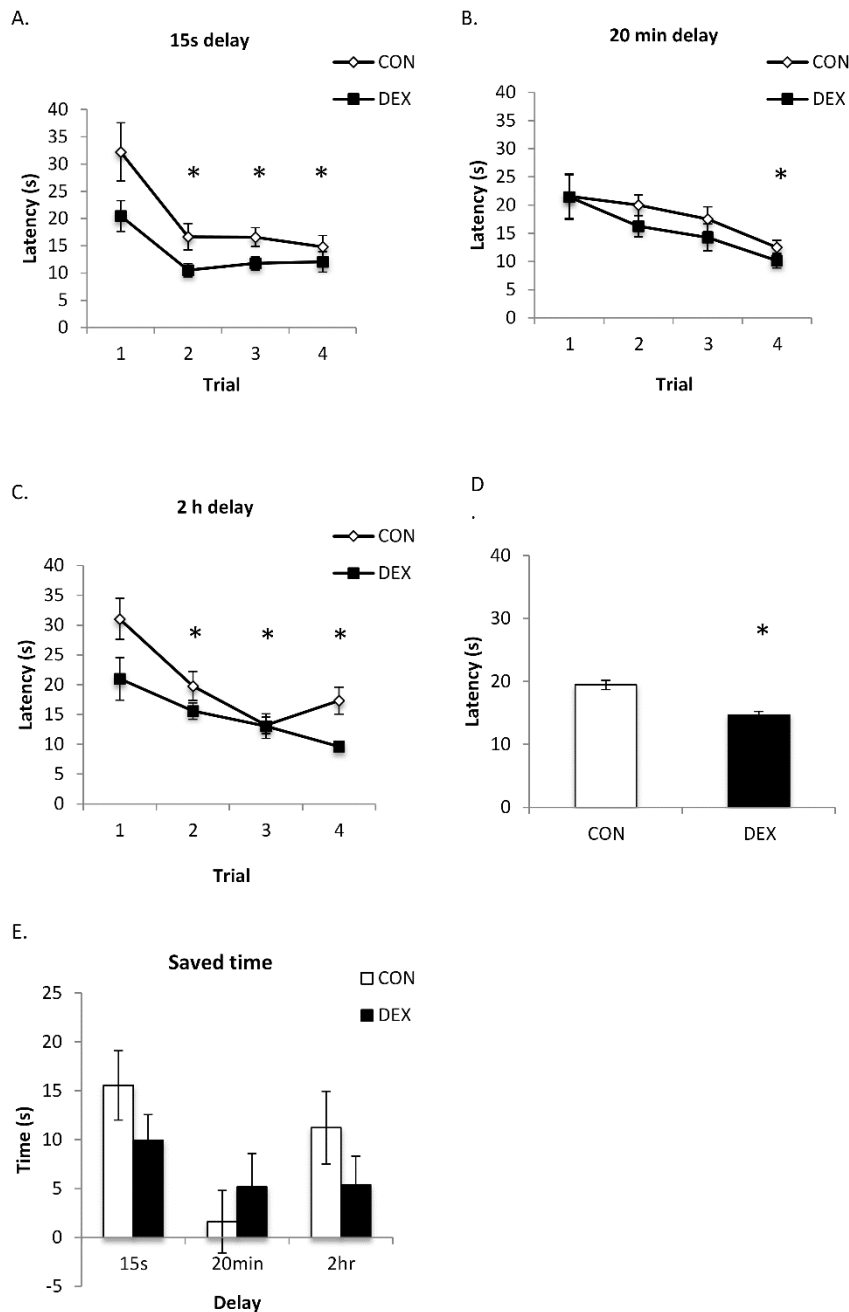


Figure 4.4 Performance during the delay phase of a DMTP task

There was a main effect of Group, such that Dex animals were significantly faster than controls to find the platform ($P=0.041$) (D). There was no main effect of Delay or any interactions between Group and Delay. However there was a significant interaction between Delay and Trial, such that all animals had significantly shortened latencies on the second trial during the 15s (A) and 2 h delay (C), whereas there was only a significant improvement on the forth trial during the 20 min delay (B). Saved time from trial 2 to trial 1 was equal between different groups and delays (E). Data are presented as mean \pm S.E.M. $n=12/\text{group}$. * $P < 0.05$ versus trial 1 (A-C); * $P < 0.05$ versus control (D).

Probe trials

There were no significant differences between the two experimental groups in terms of total amount of time spent around all the 28 platform locations ($F_{1, 8.52}=1.61$, $P=0.239$), suggesting there were no deficits in learning the possible platform locations between the two groups.

Probe trial data was analysed as the relative time spent near the most recently trained platform compared to the total amount of time spent near the other seven related locations, sitting at the vertices of an octagon. There was no significant effect of Group ($F_{1, 8.332}=1.65$, $P=0.23$), no significant effect of Delay ($F_{2, 44}=0.59$, $P=0.558$) and no interaction between Group and Delay, ($F_{2, 44}=0.48$, $P=0.621$). The average probability of swimming around the training platform location was 0.245 ± 0.140 S.D. for all animals, above the chance level, which was 0.125 for our analysis. (Figure 4.5)

Locomotor activity

There were no significant differences between the groups in average swimming speed ($F_{1, 8.01}=0.68$, $F=0.432$) or amount of time spent near the wall during the whole experiment period, ($F_{1, 7.53}=2.34$, $P=0.167$).

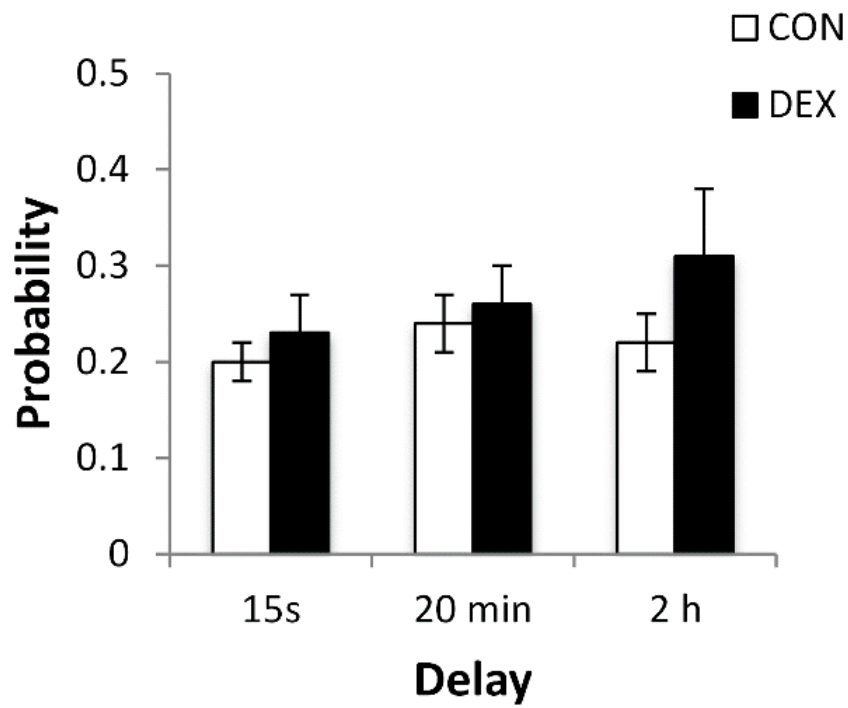


Figure 4.5 Performance on probe trials of a DMTP task

Both groups of animals showed similar preferentiality for the most recently trained platform location after any of a 15s, 20 min or 2 h delay. Data are presented as mean \pm S.E.M. n=12/group.

4.3.3 ID/ED attentional set-shifting

Discrimination learning

Figure 4.6 shows the number of trials to criteria on each of the discriminations. On average a rat took 10 trials to learn the SD. All animals demonstrated equal performance on each of the discriminations, as there was no significant effect of Group ($F_{1,10.55}=3.2$, $P=0.102$) or any interaction between Group and Discrimination, ($F_{6,126.1}=0.71$, $P=0.639$).

Reversal learning

There was a significant effect of Phase ($F_{1,136.2}=14.13$, $P=0.0003$), such that all rats required significantly more trials to learn the reversal compared to initial acquisition. There was no effect of Group on reversal learning, ($F_{1,10.85}=3.15$, $P=0.104$).

ID/ED shifts

The sequence of perceptual dimensions had no effect on performance, ($F_{1,32.4}=0.03$, $P=0.859$). All animals demonstrated equal performance on ID and ED shifts, as there was no main effect of Group ($F_{1,11.85}=0.002$, $P=0.969$), no main effect of Discrimination (ID, ED), ($F_{1,26.69}=0.80$, $P=0.378$) and no interactions between the two factors, ($F_{1,26.69}=0.38$, $P=0.541$).

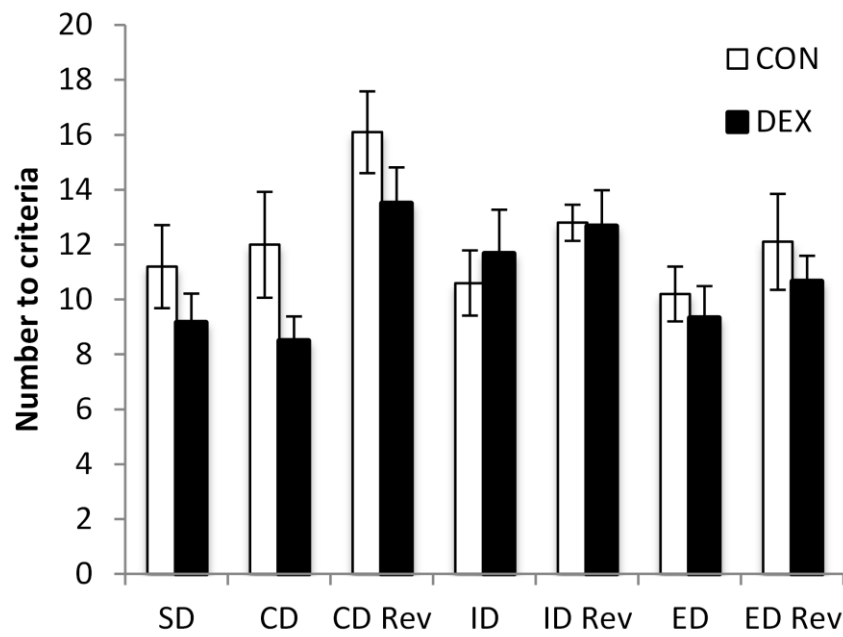


Figure 4.6 Performance on an ID/ED attentional set-shifting task

Both groups of animals had equal performance on any of the seven discriminations. Data are presented as mean \pm S.E.M. n=10-12/group.

4.4 Discussion

The experimental findings of my current study can be summarized as: a) prenatal Dex treatment during the last week of gestation in rats induced a transient deficit in spatial short-term memory in the adult offspring; b) prenatal exposure to Dex had no effect on spatial reference memory and working memory in the adult offspring; c) prenatally Dex-exposed rats had shorter latencies in the reversal learning and DMTP tasks; d) there were no effect of prenatal Dex exposure on ID/ED attentional set-shifting.

From the spatial reference memory task, prenatally Dex-treated rats demonstrated a transient deficit in locating the hidden platform on the first day of initial training. However, they were able to improve their performance and catch up with controls on the following day, indicating that their spatial reference memory was not affected. This was further supported by probe trial measures, in which all animals showed equal performance in their preferentiality to swim near the training platform on the second day of training. The current result does not support some previous studies in which offspring of stressed dams were found to have deficits in spatial reference memory (discussed in 4.1), however the current study used a different rat strain from all these studies which might account for the apparent discrepancies. In addition, experimental findings have been mixed in this field, with differences between studies potentially arising due to variations in animal strains, regimens of prenatal stress, ages and sexes of the offspring (Markham et al., 2010, Szuran et al., 2000). There is also evidence that task-related intensity of stress such as the water temperature may affect water maze findings, for example prenatal stress induced impairments in water maze performance are only observable when tested under cold water but not warm water (Szuran et al., 1994, Szuran et al., 2000).

My results also revealed a tendency that Dex-programmed animals were faster in locating the new platform position on the first day of reversal. This result indicates that prenatally Dex-

exposed animals are faster in responding to the immediate shift in experimental rules. However, this effect was only short-lasting and both groups of animals performed equally on the probe trial 24 hours after their first exposure to the new platform location. In addition, the percentage of time they spent near the old platform location was substantially reduced and was equivalent to chance searching, suggesting that both groups of animals were able to inhibit the previously rewarded response and adapt to the newly acquired information on the second day of reversal. Taken together, these results suggest that prenatal Dex treatment had no sustained effect on hippocampus-related spatial reference memory, but induced a transient deficit in processing short-term spatial memory and a tendency towards early facilitation of reversal learning in the adult male offspring.

The results on reversal learning indicated possible alterations in PFC-associated cognitive function (Debruin et al., 1994, Lacroix et al., 2002). I therefore carried out a second water maze test, the DMTP task, which assesses for higher cognitive function preferentially related to PFC, including working memory and behavioural flexibility. The working memory is characterized by delay-dependent or delay-independent impairments. Working memory deficits are delay-dependent, which are more explicit at longer delays when the load of working memory increases. By contrast, delay-independent deficits indicate deficits not specifically related to working memory, including sensorimotor function, motivation and the general procession of the procedural rules of the task (Dunnett et al., 1990, Dunnett et al., 1989). The behavioural flexibility assessed in the DMTP task is similar to that involved in the reversal learning, including shifts in responses to stimulus-related spatial information. However the reversal learning involves only one rule-switch at the reversal, whereas the DMTP task intensified the shifts in task-requirement to involve daily changes of platform locations. Thus, if there were any potential group differences in behavioural flexibility, as indicated by the reversal learning, it might become more explicit in the DMTP task.

Interestingly, my current results revealed a significant treatment effect on DMTP performance, with prenatally Dex-programmed rats having shorter latencies to platform over the whole experiment. The faster acquisition in prenatally Dex-exposed rats indicates increased flexibility in responding to changes in environmental circumstances. This result is consistent with the reversal learning, and the behavioural difference did become more explicit in the DMTP task as the task requirement intensified to involve much more frequent behavioural shifts.

However, my current results revealed no main effect of Delay or any interaction between Group and Delay in latency time to platform and saved time from trial 2 to trial 1, suggesting that the difference in performance were caused by delay-independent deficits, rather than delay-dependent deficits. As discussed above, this result indicates that working memory may not be affected by prenatal Dex-treatment, which was further supported by their equivalent performance on probe trials after any time of a delay. Instead, the current results suggest that the increased behavioural flexibility in prenatally Dex-exposed rats may involve non-mnemonic processes interfering with learning and executive function, including sensorimotor function, motivation, attention, impulsivity and response inhibition.

Since all animals were able to use spatial visual cues to navigate around the water maze, any global deficits in visual perception or swimming ability can be excluded. There were no group differences in locomotion, as measured by swimming speed. Likewise, there were no deficits in their primary motivation to escape from the water maze, as all animals were driven to find the platform irrespective of how long it takes.

A possible contribution of impaired attention and impulsivity cannot be excluded, such that prenatally Dex-exposed rats may respond faster due to a lack of sustained attention or endogenous response inhibition, resulting in an increase in spontaneous response, thereby

yielding a 'facilitation' during the DMTP task. There is evidence from previous study that a lack of response inhibition leads to an increase in nondiscriminated response, and thus results in 'false facilitation' during reversal of a spatial discrimination task (Salazar et al., 2004). In my current DMTP task, if the faster responses of Dex-programmed animals were driven by increased impulsivity and were nondiscriminated, we might observe less response latency and more spontaneous errors, which cannot be tested under the current DMTP protocol, and additional experiments would be required to elucidate this further.

A less perseveration to the previous rewarded response could also account for the increased behavioural flexibility in prenatally Dex-exposed animals. However the current DMTP task do not give direct evidence for possible differences in perseveration tendencies and additional experiment might be required to investigate this issue further. Overall the current evidence suggests that prenatally Dex-treated animals are more flexible in shifting their responses to changes in stimulus-reward contingencies, potentially linked to increased cognitive flexibility and responsive adaptability.

The behavioural flexibility assessed on the above two water maze tasks involves only intra-dimensional shifts in response to changes in task requirements. I took this further by carrying out a next ID/ED attentional set-shifting task on another set of animals, which assesses both intra-dimensional (Revs, ID) and extra-dimensional (ED) behavioural flexibility within and between perceptual dimensions. My current results suggest that there was no overall group difference on ID/ED performance, thus prenatal Dex exposure had no effect on behavioural flexibility in terms of reversing a stimulus-reward association within a particular perceptual dimension, as well as attentional set-shifting between perceptual dimensions. The sequence of shifting (odour to medium versus medium to odour) had no effect on performance, thus there were no differences regarding the ease in making the two kinds of shifts. Previous studies report learning is faster within the same dimension (ID) compared to ED in Lister Hooded rats

(Birrell and Brown, 2000) and mice (Garner et al., 2006). However, in my current experiment all animals including controls performed equally well on ID and ED shifts, suggesting that the ID/ED task may not be a sensitive measure for cognitive set-shifting in Wistar rats.

Taken together, my current results suggest that prenatal exposure to Dex affected cognition in adult male offspring in rats. There was little evidence that prenatal Dex treatment had any effect on mnemonic processes, except for a transient deficit in processing spatial short-term memory. Prenatal Dex-treatment had no effect on attentional set-shifting within and between perceptual dimensions, however it facilitated behavioural flexibility in responding to changes in stimulus-reward associations within the water maze tasks, potentially associated with altered neural networks involving the hippocampus and PFC.

Chapter 5

Mechanisms of programming:

Possible implications for behaviour

5.1 Introduction

5.1.1 Introduction

Human and animal studies suggest that prenatal stress influences the development of the brain and the organization of behaviour, characterized by a number of neuroendocrine, behavioural and neurobiological changes in the offspring (detailed in 1.1.2). It has been hypothesized that the aversive effect of prenatal stress on brain structure and function may be partly mediated by altered gene expression in the brain (discussed in 1.1.2 and 1.4.3.1).

In previous chapters, I have reported that prenatal administration of Dex during the last week of gestation in rats had no observable effect on HPA axis function and unconditioned fear and anxiety-like behaviour in the adult male offspring; however, prenatally Dex-exposed rats demonstrated different performance in a contextual fear conditioning test and water maze tasks, suggesting altered cognition associated with hippocampus and PFC function. The molecular basis underlying these behavioural phenotypes is however not very well understood.

In this chapter I aimed to find out if the long-term effects of prenatal Dex treatment on offspring behaviour are correlated with altered gene expression in the brain. Based on my current behavioural findings, I am prompted to select candidate genes that are related to behaviour strategies and memory function. Three candidate genes were chosen from literature review, including glucocorticoids receptors (*GR* and *MR*) and brain-derived neurotrophic factor (*Bdnf*). One candidate gene was chosen from the result of a previous Affymetrix microarray from our group, early growth response 1 (*Egr1*).

Glucocorticoids receptors

The stress hormones act via two receptor types in the brain: the low affinity glucocorticoid receptor (GR) and the high affinity mineralocorticoid receptor (MR). Both receptors act as transcription factors and regulate different aspects of fear, anxiety and cognitive behaviour (Brinks et al., 2009, de Kloet et al., 1999). For example, it has been suggested MR mediates the regulation of basal CORT levels during circadian rhythm, whereas GR occupancy is involved in the negative feedback of a hormonal stress response. In terms of behavioural functions, brain MR is thought to mediate acute fear-related behaviour and fear extinction, whereas GR-occupation is thought to facilitate fear potentiation and consolidation of fear-related memory (Korte, 2001, Brinks et al., 2009). In addition, brain MR and GR are involved in different aspects of spatial learning, such that MR is necessary for response selection and behavioural strategy, whereas GR is involved in consolidation of acquired information (Oitzl and de Kloet, 1992, Berger et al., 2006).

Bdnf

BDNF is a member of the neurotrophin family, and is expressed in the brain at different developmental stages (Chao, 2003). In addition to its involvement in neurodevelopment, *BDNF* signalling is critical for synaptic plasticity in adults and plays an important role in long-term potentiation (LTP) in the hippocampus (Leal et al., 2014). There is evidence that prenatal stress in rats leads to dysregulation of corticostriatal *Bdnf* expression associated with permanent alterations in brain function (Fumagalli et al., 2004). In addition, *BDNF* has been implicated in a range of neuropsychiatric disorders (Green et al., 2011a, Rakofsky et al., 2012, Halepoto et al., 2014).

Egr1

Egr1 is selected from the Affymetrix microarray result from a previous study of our group. Total RNA was extracted from the adult male prefrontal cortex of the prenatal Dex treatment rat model and 8 samples of good quality total RNA samples (n=CON:4; DEX:4) were selected for Affymetrix microarray, with analysis taken place at the Wellcome Trust Clinical Research Facility in Edinburgh. According to the microarray, *Egr1* was up-regulated in the prefrontal cortex of prenatally Dex-treated rats with a fold change of 1.1767, $P=0.0176$. *Egr1* is one of the immediate early genes (IEGs) that act by regulating the expression of downstream genes in response to neuronal activity (Sukhatme et al., 1988). It has been suggested that the expression of IEGs including *Egr1* plays a role in synaptic plasticity required for memory formation and consolidation processes (Guzowski et al., 2001).

In the context of the current study, these four genes represent important candidates for understanding the molecular basis of the behavioural phenotypes induced by prenatal Dex administration. I therefore investigated the mRNA expression of these four candidate genes by qPCR in dissected brain regions of the prenatal Dex exposure rat model.

In addition to this, our group also carried out a genome-wide next generation RNA-sequencing (RNA-Seq) experiment to provide a complete and unbiased picture of the transcriptome of the adult PFC after prenatal Dex treatment. Validation of the RNA-Seq results by qPCR revealed a prenatal Dex treatment effect to increase the mRNA expression of activity-regulated cytoskeletal-associated protein (*Arc*, also termed *Arg3.1*) in the adult PFC (fold change 1.70, $P<0.01$). *Arc* is another IEG (Lyford et al., 1995) together with *Egr1* that have been implicated in postsynaptic plasticity and memory formation (Korb and Finkbeiner, 2011). In rodents, *Arc* is constantly expressed at low levels under basal conditions and its expression can be further induced by neural activities in response to learning (Montag-Sallaz and Montag, 2003). ARC protein plays a critical role in LTP and memory consolidation in rats (Guzowski et al.,

2001). There is evidence that *Arc* expression is regulated by DNA methylation (Dyrvig et al., 2012). In the current study, I also aimed at investigating the possible role of DNA methylation in mediating the prenatal Dex exposure effects on *Arc* transcription.

5.1.2 Hypothesis and aim

I hypothesise that the prenatal glucocorticoid exposure effects on brain function and behaviour may in part be mediated by altered gene expression in the brain. The aim of this study is to explore possible molecular mechanisms underlying the prenatal glucocorticoid effects on behaviour.

In this chapter I will study the mRNA expression of four candidate genes that are known to be involved in behaviour and memory function in the brain of prenatal Dex exposure rat model. I will also study the effects of prenatal Dex exposure on the transcriptome of the adult PFC by a hypothesis-free RNA-seq study.

5.2 Methods

5.2.1 Animals

30 female Wistar rats were housed individually and time mated with a stud Wistar male. The presence of a vaginal plug was checked daily in the morning. Following vaginal plugging (denoted as E1) pregnant dams were randomly assigned to Dex or vehicle (Con) treatment. From E15-E21 inclusive, dams were given a daily subcutaneous injection of either Dex (100µg/kg body weight) or an equivalent volume of vehicle (4% ethanol, 0.9% saline) (0.5ml/kg).

At E20, 12 pregnant females (n=6,6) were killed by carbon dioxide asphyxiation. The fetuses were isolated from the uterus and killed by decapitation. Fetal heads were snap frozen on dry ice and stored at -80°C. A limb of the fetus was also taken to enable later genotyping for determination of sex, since this can be difficult to determine prenatally.

The rest of the pregnant females were allowed to deliver litters normally and after littering down (postnatal day 1), pups were weighed, sexed and litters culled back to eight per group. Litters were reared by their biological mother until three weeks of age, whereupon female offspring were culled and male pups were housed with animals from same treatment group and left undisturbed until adulthood, at which point they were sacrificed (n=CON:15, DEX:17, aged 88.88 ± 1.88 S.D. days) and the PFC, hippocampus, hypothalamus and cerebellum were collected. Each anatomical specimen was immediately frozen and stored at -80°C until use.

5.2.2 Sex genotyping for E20 samples

DNA was extracted from E20 fetal limb using DNeasy® Blood and Tissue kit (Qiagen) and followed by PCR for the SRY gene which lies on the Y chromosome to allow for identification of male fetuses by gel electrophoresis.

DNA samples were diluted to 1:10 in nuclease free water. Samples were run together with a positive control (SRY positive male control), a negative control (SRY negative female control) and a non-template control (nuclease free water) to allow identification of male-specific chromosome and to ensure no contamination occurred. Primer mix (2µM) was made from stock solutions (100µM) with 10µl forward primer (actgttcaagcagtcagccg) and 10µl reverse primer (ctccatgaacttggggtc) in 480µl nuclease free water. The PCR reaction conditions comprised rapid heating to 95°C for 10 minutes for denaturation followed by 40 cycles of a denaturation step at 95°C for 20 seconds, annealing at 58°C for 20 seconds, and elongation at 72°C for 20 second. The samples were then cooled to 72°C for 7 minutes for completion in a G-Storm thermal cycler.

	Volume
Master Mix	10 μ l
Solution Q	2 μ l
Primer Mix (2 μ M)	2 μ l
DNA (1:10)	1 μ l
Nuclease free water	5 μ l

Table 5.1 PCR recipe for sex genotyping

5.2.3 Quantitative real-time PCR

The whole brains from male E20 embryos were removed from the skull and the frontal lobes were used for simultaneous RNA/DNA extraction (as described in 2.3.2). For the adult brain tissues, gene expression was examined in dissected brain regions. Right dorsal PFC was used for simultaneous RNA/DNA extraction (as described in 2.3.2), and right hippocampus was used for total RNA extraction following the RNeasy® mini kit (Qiagen) protocol.

The amount of total RNA was determined using the Qubit® RNA HS Assay Kit (Invitrogen). 800ng of total RNA (n=CON:10, DEX:8 for E20 frontal lobe; n=CON:9, DEX:10 for adult PFC; n=CON:10, DEX:10 for adult hippocampus) was treated with DNase (Promega) and then reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) to produce cDNA. qPCR was performed to examine gene expression by TaqMan™ (Applied Biosystems) or UPL (Roche) assays with the addition of LightCycler® 480 Probes Master (Roche). Expression of genes was normalized to hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) for adult hippocampus samples, and to the average of the three housekeeping genes, *Hprt*, glucose-6-phosphate dehydrogenase deficiency (*G6pd*) and TATA box binding protein (*Tbp*) for the E20 and adult PFC samples.

5.2.4 Next generation RNA sequencing

As described in 5.2.3, total RNA was extracted from right dorsal PFC samples (n=CON:9, DEX:10) according to the simultaneous RNA/DNA extraction protocol detailed in 2.3.2. A RNeasy® plus mini kit was used (Qiagen) instead of a RNeasy® mini kit (Qiagen) and manufacturer's protocols followed. Final product was eluted in a final volume of 30µl RNase-free water. Total RNA quality was assessed by gel electrophoresis for purity and also on an Agilent Bioanalyser (Agilent Technologies) for integrity. The presence of a clean thicker 28S and a thinner 18S ribosomal RNA bands (at about 2:1 ratio) without a DNA band on the gel, and an RNA Integrity Number (RIN) number above 9.0 on Agilent Bioanalyser was considered satisfactory.

8 samples of best quality (n=CON:4, DEX:4) were selected for next generation RNA-Seq. After quantification of RNA concentrations by using the Qubit® RNA HS Assay Kit, 1µg of total RNA (100ng/ul) was prepared for each sample. Samples were then transported on dry ice to Holland where the next generation RNA-Seq was performed by BaseClear (Leiden) and data analysis was undertaken by the MRC Computational Genomics Analysis and Training (CGAT) facility (MRC Functional Genomics Unit at the University of Oxford).

5.2.5 DNA methylation analysis

Bisulfite-treated DNA samples from E20 frontal lobe (n=CON:10, DEX:10) and adult PFC (n=CON:8, DEX:9) were amplified using self-designed primers that targeted either the CpG-rich region within the *Arc* promoter or intragenic regions of the *Arc* gene. Schematic diagrams of the examined regions are demonstrated in Figure 5.1.

1µl of bisulfite converted DNA was used as template in a 20µl PCR reaction mixture with the addition of primers and master mix (Table 5.2). The PCR program comprised rapid heating to 95°C for 10 minutes for denaturation followed by 45 cycles of a denaturation step at 95°C for 20 seconds, annealing at 56°C for 20 seconds and elongation at 72°C for 20seconds. The samples were then stored at 72°C for 7 minutes for completion. Methylation of DNA was measured by Pyrosequencing using the PyroMark Q24 system (Qiagen) as described in 2.3.5.3.

	Volume
AmpliTaq Golden Master Mix	10µl
Primer Mix (2µM)	4µl
DNA	1µl
Nuclease free water	5µl

Table 5.2 Recipe for Pyrosequencing PCR

tccgggtgggagggcgcgagcagagcacattagtcactccgggctgt
 gaaggggaggggtccttgagggcaccgcggaaggggagcgagtag
 gcgcgggagagggcggggacctgcggcaggagagggcgcgggcggg
 ctctggcgcgagcctggggcgcgccaatgggagccagggctccgc
 gagctgccgcccacggggccccgcgcagcataaaaagccgcccgtgg
 cggcttttagtgAGTGCTCTGGCGAGTAGTCCTCCCTCAGC
 CGCAGTCTCTGGGCCTCTTCAGCTTGAGCGGCGGCGAG
 CCTGCTACACTCGCTAAGCTCCTCCGGCACCGCGCACTT
 GCCACTGCCACTGCCGCTTCGCGCCCCGCTGCAGCCGCC
 GGCTCTGAATCCTTCTGGCTTCCGCCTCAGAGGAGTTCT
 TAGCCTGTCCCGAACCGTAACCCCGGCGAGCAG**ATGGA**
 GCTGGACCATATGACGACCGGCGGCCTCCACGCCTACC
 CTGCCCCGCGGGGTGGGCGCGGCCGCAAACCCAATGTG
 ATCCTGCAGATTGGTAAGTGCCGAGCTGAGATGCTGGA
 GCACGTACGGAGGACCCACCGGCATCTGTTGACCGAAG
 TGTCCAAGCAGGTGGAGCGAGAGCTGAAAGGGTTGCAC
 AGGTCGGTGGGCAAGCTGGAGAACAACCTTGGACGGCTA
 TGTGCCACGGGCGACTCACAGCGCTGGAAGAAGTCCA
 TCAAGGCCTGTCTCTGCCGCTGCCAGGAGACCATCGCC
 AACCTGGAGCGCTGGGTCAAGCGTGAGATGCACGTGTG
 GAGGGAGGTCTTCTACCGTCTGGAGAGGTGGGCGGACC
 GCCTGGAGTCCATGGGCGGCAAGTACCCAGTGGGCAGC
 GAGCCGGCCCGCCACACTGTCTCTGTAGGTG

Figure 5.1 Schematic diagram of the examined regions of *Arc*

The promoter region is represented in lower-case and the intragenic region is represented in upper-case. The start codon ATG (underlined in bold) indicates the start of the coding DNA sequence (CDS). The sequences being analysed are shaded in grey, and are picked up by assays *Arc_P1*, *Arc_P2*, *Arc_GB1* and *Arc_GB2*, respectively.

5.2.6 Statistical analysis

Data were analysed using GLMs (JMP statistical software, SAS Institute, Cary, NC, USA). All data were checked for homogeneity of variance and normality of distribution. All statistical models investigated the main effect of Group. Litter (nested within group) was fitted as random variable to account for the use of more than one animal per litter. Data were presented as mean value per group \pm S.E.M. Results were considered significant if $p < 0.05$.

5.3 Results

5.3.1 Candidate gene expression in the brain

Figure 5.2 shows the expression of candidate genes in the E20 frontal lobe (A), adult PFC (B) and hippocampus (C). The expression of *Egr1* was significantly decreased in the E20 frontal lobe of Dex-treated embryos, ($F_{1,8.19}=10.39$, $P=0.012$). In the adult brain, there was an effect of prenatal Dex-exposure to increase the expression of *Egr1* in the PFC ($F_{1,11.15}=8.26$, $P=0.015$), and a trend towards an increased expression of *Egr1* in the hippocampus of prenatally Dex-exposed rats, ($F_{1,14.51}=4.18$, $P=0.060$). There was no effect of prenatal Dex-exposure on *Bdnf* expression in the brain. There were no significant differences between the groups in *GR* expression in the E20 frontal lobe and adult PFC, whereas in the adult hippocampus there was an effect of prenatal Dex treatment to increase the expression of *GR*, ($F_{1,14.65}=5.59$, $P=0.032$). The expression of *MR* was too low in the E20 frontal lobe for formal analysis. In the adult brain, *MR* expression was unaltered in the hippocampus but significantly increased in the PFC of prenatally Dex-treated rats, ($F_{1,13.57}=6.45$, $P=0.024$).

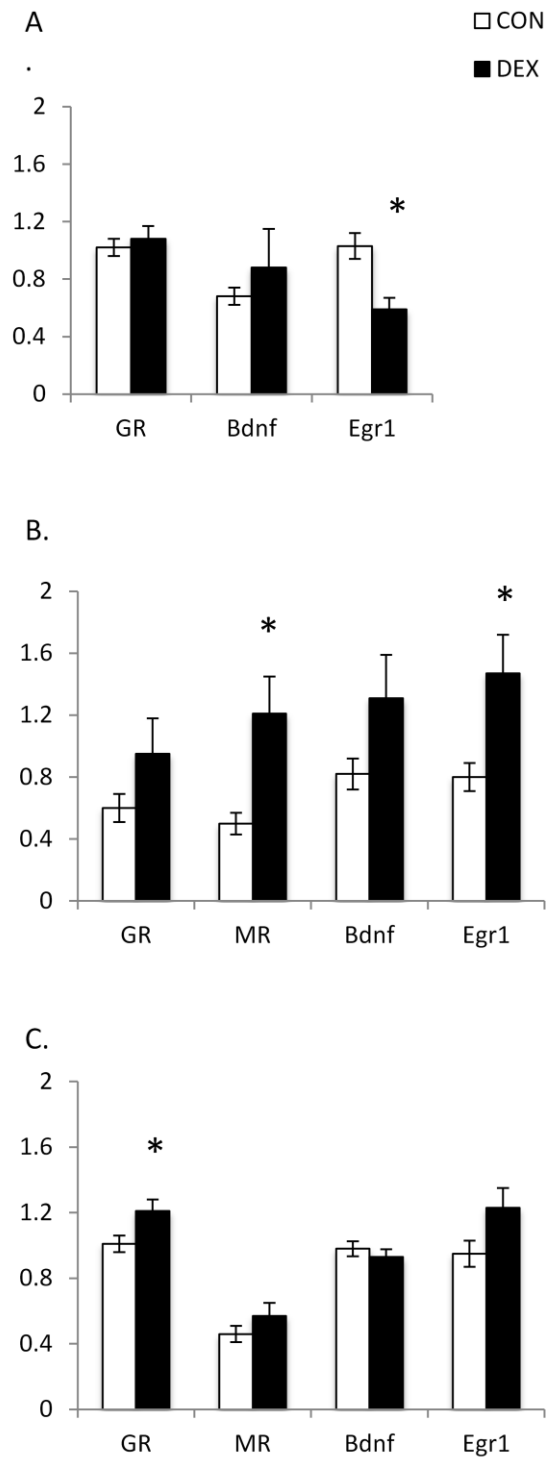


Figure 5.2 Candidate gene expression in the brain

There was an effect of prenatal Dex treatment to decrease the expression of *Egr1* in the E20 frontal lobe (A) while increase its expression in the adult PFC (B). There was an increased *MR* expression in the adult PFC (B) and an increased *GR* expression in the adult hippocampus (C) of prenatally Dex-exposed animals. Data are presented as mean \pm S.E.M. n=8-10/group. * $P < 0.05$ versus control.

5.3.2 Validation of results from next generation RNA sequencing

The next generation RNA-seq data were processed by Dr Andreas Heger through the MRC CGAT programme. Three differential analytical tools were used to identify differences in mRNA levels of individual genes between control and experimental samples including DESeq, edgeR and Cuffdiff. Each of these methods generated different lists of regulated genes and was associated with high rates of false positive (Cuffdiff) and false negative (DESeq) discovery, however in general these methods had fairly good agreement, and most of the genes considered differentially expressed were shared between methods.

One problem with RNA-seq was its small size number with $n=4$ per group, and the results in general did not cluster very well by group. I thus identified a list of genes from those that were consistently identified as differentially expressed whichever statistical method was used (Table 5.3 and Table 5.4), and thereafter validated the mRNA expression on a larger number of adult PFC samples ($n=\text{CON}:9, \text{DEX}:10$) using qPCR. The increased expression of *Arc* in the PFC of prenatally Dex-exposed rats was validated by qPCR ($F_{1,11.25}=7.47, P=0.019$), however the expression of other genes on the list was not validated. (Figure 5.3)

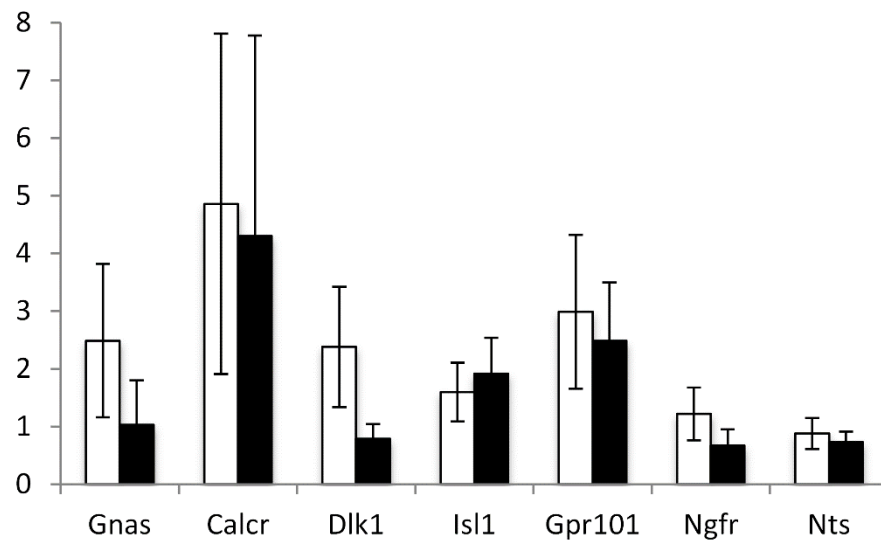
Gene name	Description	Gene function	Fold_change	P value
<i>Gnas</i>	GNAS complex locus	maternally imprinted gene, circadian gene, involved in sleep, cognition, fear conditioning consolidation (Lassi et al., 2012)	-5.902371761	7.66E-18
<i>Calcr</i>	calcitonin receptor	brain-specific maternally imprinted gene, possibly involved in pain modulation, serotonergic pathways, related to autism (Hoshiya et al., 2003)	-4.840227599	4.93E-15
<i>Dlk1</i>	delta-like homolog 1	paternally imprinted gene	-1.24981	0.00343077
<i>Isl1</i>	ISL LIM homeobox 1	expressed in forebrain, involved in cholinergic pathway, regulates cognitive processes (Cho et al., 2014)	-2.74937	0.0005
<i>Gpr101</i>	G protein-coupled receptor 101	expressed predominantly in the brain (limbic, autonomic and sensory areas), suggesting a role in behaviour, motivational drive and attentional state (Bates et al., 2006)	-2.458700914	1.18E-12
<i>Ngfr</i>	nerve growth factor receptor	involved in nerve growth	-2.301546511	5.80E-10
<i>Nts</i>	neurotensin	modulates dopaminergic neurotransmission (Binder et al., 2001)	-2.25287461	2.69E-13

Table 5.3 Top list of down-regulated genes from adult PFC RNA-Seq

Gene name	Description	Gene function	Fold_change	P value
<i>Arc</i>	activity-regulated cytoskeleton-associated protein	involved in synaptic plasticity (Lyford et al., 1995)	1.70081	5.00E-05
<i>Gfral</i>	GDNF family receptor alpha like	involved in maturation and survival of differentiated dopaminergic neurons (Airaksinen et al., 2006)	1.65832	0.0007
<i>Th</i>	tyrosine hydroxylase	involved in catecholamine biosynthesis	1.27928801	0.002060492
<i>Qrfpr</i>	pyroglutamylated RFamide peptide receptor	involved in regulating neuroendocrine function, feeding behaviour and blood pressure (Chartrel et al., 2011)	1.136553117	2.45E-05
<i>Rspo1</i>	R-spondin 1	possibly involved in energy homeostasis (Li et al., 2014)	1.185527549	1.64E-05

Table 5.4 Top list of up-regulated genes from adult PFC RNA-Seq

A.



B.

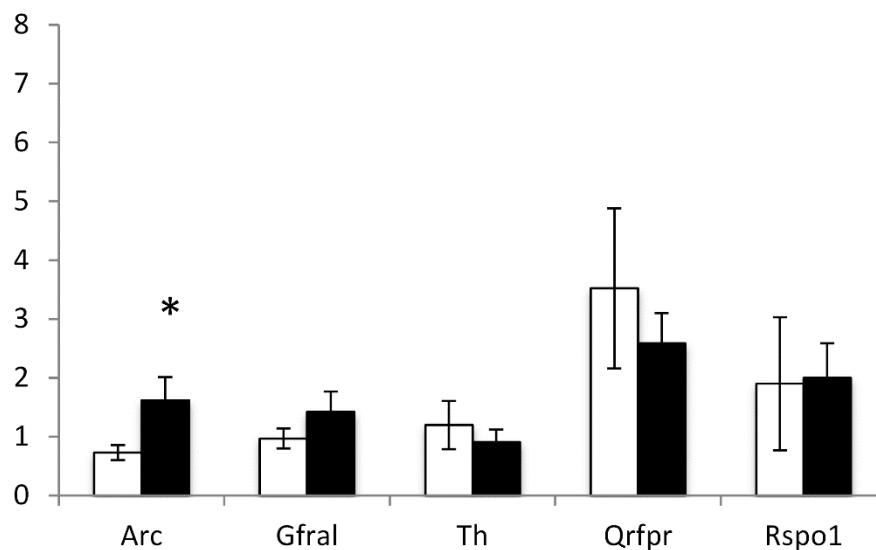


Figure 5.3 Validation of differentially expressed genes from RNA-Seq in adult PFC using qPCR

The expression of a number of down-regulated genes from RNA-Seq was not validated by qPCR (A). The increased expression of *Arc* in the PFC of prenatally Dex-exposed rats was validated by qPCR ($P=0.019$), however the expression of other up-regulated genes from RNA-Seq was not validated (B). Data are presented as mean \pm S.E.M. $n=9-10/\text{group}$. * $P < 0.05$ versus control.

5.3.3 *Arc* expression in the brain

Since there was an increased expression of *Arc* in the adult PFC of prenatally Dex-treated rats, I further investigated the expression of *Arc* in the E20 frontal lobe and adult hippocampus samples. There was no effect of prenatal Dex treatment on *Arc* expression in the E20 frontal lobe. However, there was an increased *Arc* transcription in the adult hippocampus of prenatally Dex-exposed rats, ($F_{1,10.93}=6.66$, $P=0.026$). (Figure 5.4)

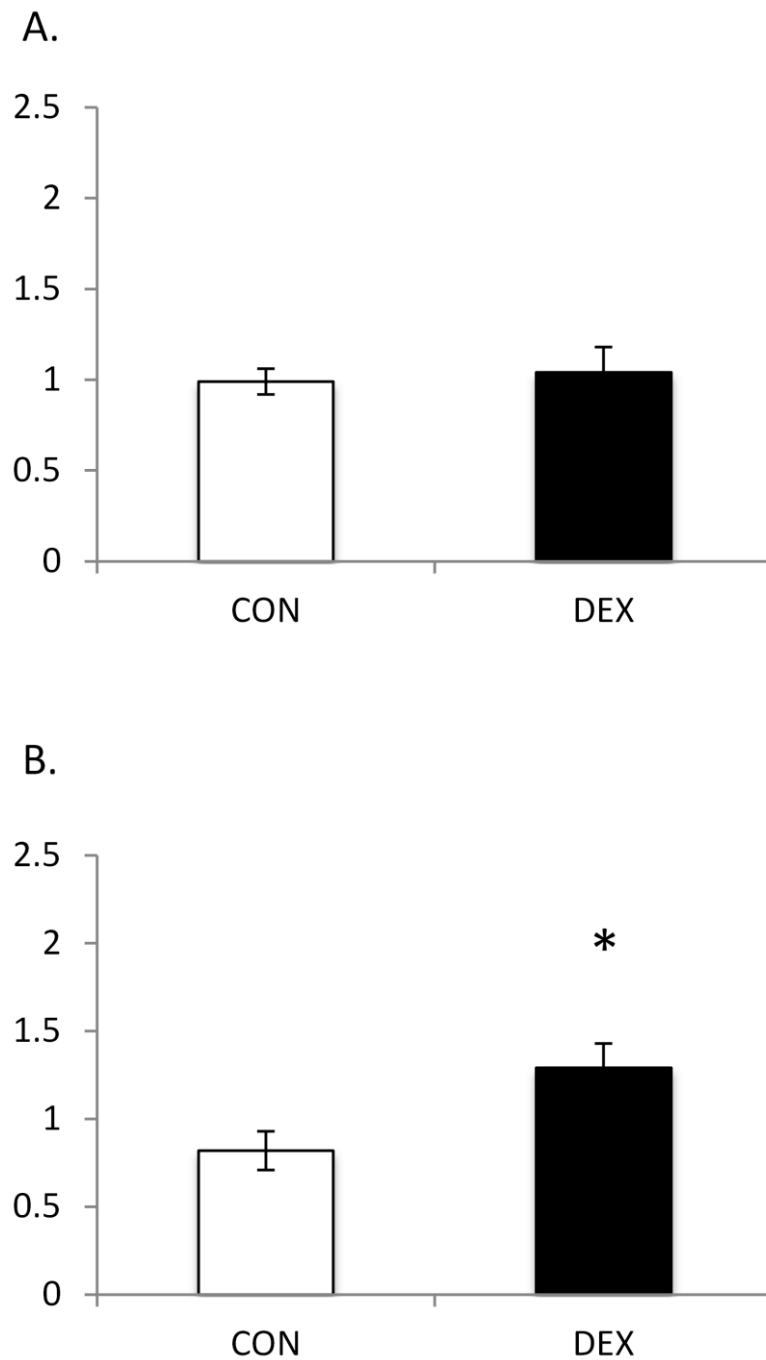


Figure 5.4 *Arc* expression in the brain

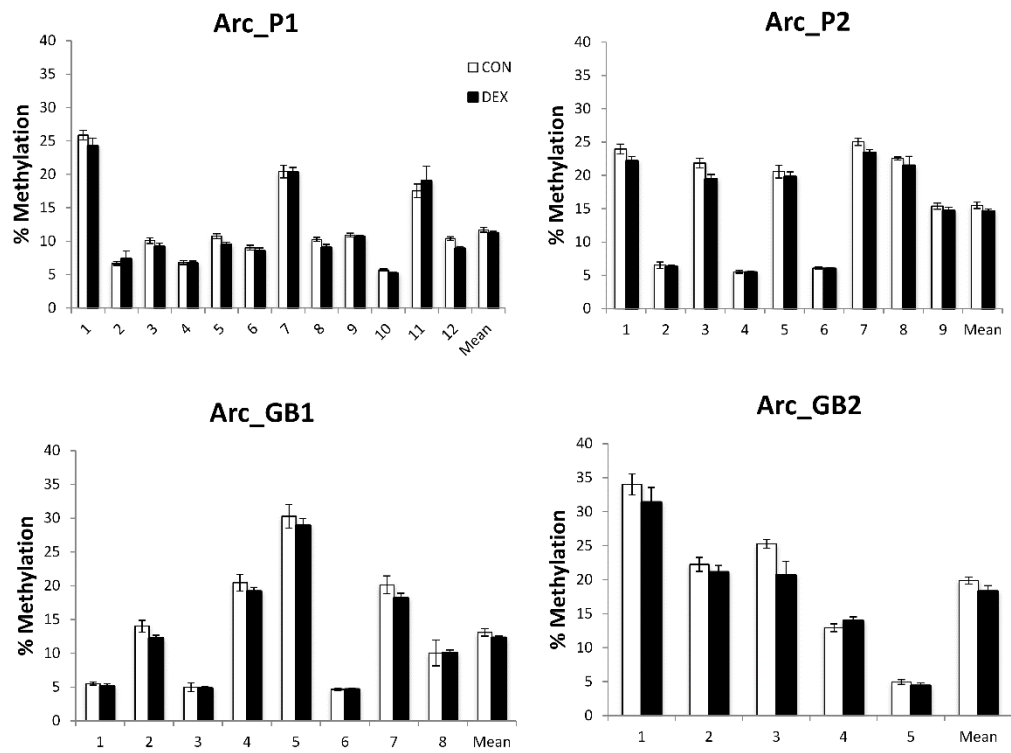
There were no differences between the groups in *Arc* expression in the E20 frontal lobe (A); *Arc* expression was significantly increased in the adult hippocampus of prenatally Dex-exposed rats ($P=0.026$) (B). Data are presented as mean \pm S.E.M. $n=8-10$ /group. * $P < 0.05$ versus control.

5.3.4 DNA methylation at the *Arc* promoter and intragenic regions

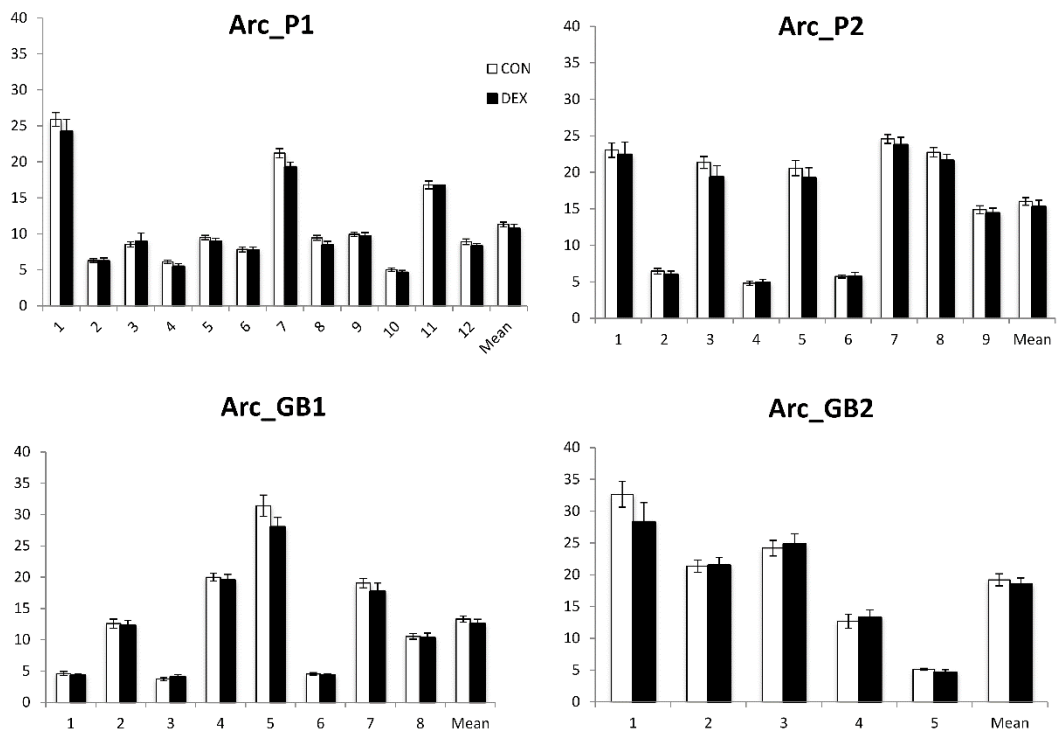
Figure 5.5 demonstrates the distribution of cytosine methylation at individual CpG sites in the analysed regions of the *Arc* gene. The efficiency of bisulfite conversion was calculated based on conversion rates of non-CpG cytosines and was around 90%.

There were no effects of prenatal Dex treatment on DNA methylation at *Arc* promoter (detected by *Arc_P1* and *Arc_P2*) and intragenic regions (detected by *Arc_GB1* and *Arc_GB2*) in the E20 frontal lobe (A) and adult PFC (B). The percentage of DNA methylation varied between 3% to 35% across analysed CpGs, and the pattern of DNA methylation was similar between the E20 frontal lobe and adult PFC (C).

A)



B)



C)

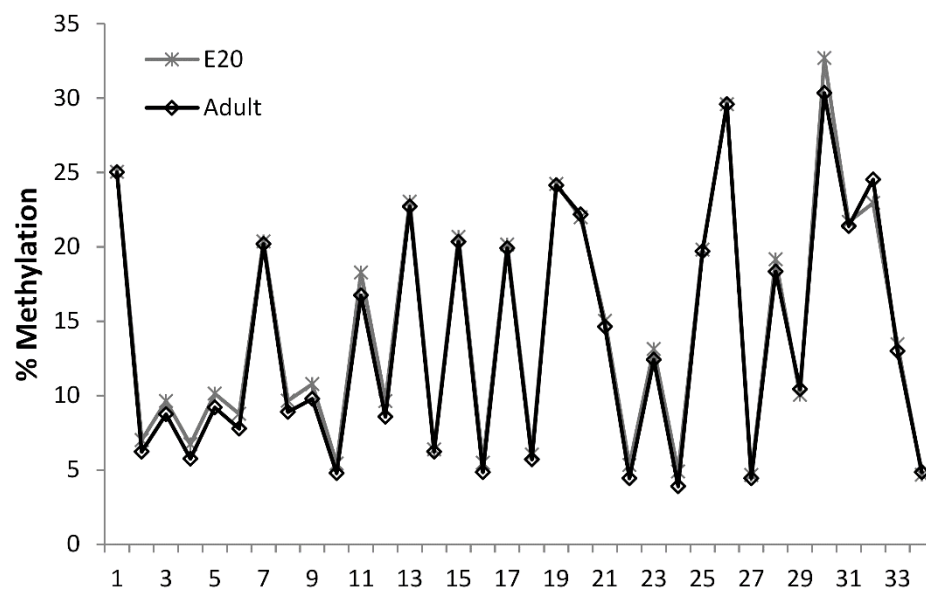


Figure 5.5 DNA methylation at the *Arc* gene promoter and intragenic regions

There were no significant differences between the two experimental groups in *Arc* methylation at the promoter and intragenic regions in either the E20 frontal lobe (A) or adult PFC (B). The DNA methylation pattern at the examined 34 CpG sites was similar between the E20 frontal lobe and adult PFC (C). Data are presented as mean \pm S.E.M. n=8-10/group.

5.4 Discussion

In the present study, I investigated possible molecular correlates of behavioural phenotypes induced by prenatal Dex exposure in rats. I examined the baseline expression of candidate genes involved in synaptic plasticity and memory formation in dissected brain regions of the prenatal Dex-exposure animal model. I also validated the results of next generation RNA-Seq on adult PFC samples by qPCR and further investigated the possible epigenetic mechanisms associated with altered *Arc* expression. My results revealed altered brain expression of genes involved in synaptic plasticity and memory formation in prenatally Dex-exposed animals, with no indication of regulation by promoter DNA methylation.

In my current experiment, there was an increased *GR* transcription in the adult hippocampus of prenatally Dex-exposed rats. This result appears to contrast with some previous studies, in which prenatal glucocorticoid exposure was found to permanently decrease *GR* expression in the hippocampus (Welberg et al., 2001, Levitt et al., 1996). However, there is evidence that prenatally Dex-exposed rats reared by control mothers are found to have increased hippocampal *GR* mRNA and enhanced glucocorticoid feedback (Brabham et al., 2000). In my current experiment, the increased hippocampal *GR* mRNA in the adult hippocampus of prenatally Dex-exposed rats could potentially due to random environmental enrichment at neonatal time. The increased *GR* expression in the hippocampus of prenatally Dex-treated rats may facilitate HPA axis feedback and is consistent with the normal basal and stressed-induced CORT response in these animals.

Prenatal Dex exposure increased the expression of *MR* in the adult PFC. This is potentially interesting considering the role of MR in behavioural adaptation and strategy (Oitzl and de Kloet, 1992). Ablation of forebrain *MR* expression in mice has been shown to result in impaired behavioural responses to changes in task-requirements in the water maze, consistent

with behavioural perseverance and stereotypy (Berger et al., 2006). In my current study, an increased *MR* expression in the adult PFC of prenatally Dex-exposed rats is potentially consistent with facilitation of PFC-associated behavioural flexibility in the reversal learning and the DMTP task (chapter 4).

My current results suggest prenatal Dex exposure had no effect on brain *Bdnf* expression. However, there was an increased expression of *Egr1* in the adult PFC, with a trend towards increased expression in the adult hippocampus of prenatally Dex-exposed rats. In contrast, *Egr1* expression was significantly decreased in the E20 frontal lobe of Dex-treated fetuses, indicating its expression may be differentially regulated at different developmental stage. As described in 5.1, *Egr1* is one of the IEGs that has been implicated in experience-dependent synaptic plasticity. My current results of altered *Egr1* expression in the E20 and adult brains suggest that prenatal stress may affect cognitive and memory processes through programming effects on synaptic development and connectivity.

In addition to candidate gene approach, I also prepared samples and sent for next generation RNA-Seq to characterize the adult PFC transcriptome of the prenatal Dex exposure model, which revealed differentially expressed genes implicated in synaptic plasticity, cognition, dopaminergic, serotonergic and cholinergic pathways. Validation of the sequencing results confirmed increased expression of *Arc* mRNA in the adult PFC and hippocampus of prenatally Dex-treated rats.

The increased expression of *Arc* in the adult brain of prenatally Dex-exposed rats is interesting. ARC protein is an important component of the N-methyl-D-aspartate (NMDA) receptor signalling complex at the postsynaptic membrane of glutamatergic synapses (Korb and Finkbeiner, 2011) and there is evidence that prenatal stress induces programming effects on

the NMDA receptor signalling complex. For example, one previous study reported that prenatal stress in rats induces significant changes in genes associated with the NMDA receptor/postsynaptic density complex (Kinnunen et al., 2003). Another group reported that prenatal stress produces learning deficits associated with impaired NMDA receptor-mediated LTP in the hippocampus (Son et al., 2006). Furthermore, schizophrenia patients were found to have an enrichment of *de novo* mutations involved in genes belonging to components of the NMDA receptor signalling complex including *Arc* (Kirov et al., 2012, Fromer et al., 2014).

In addition, an increased brain expression of *Arc* is potentially consistent with an increased brain *Egr1* expression in prenatally Dex-exposed rats. Indeed, previous studies report synchronized regulation of IEGs expression such as *Arc* and *Egr1* in rats in response to learning, and the level of *Arc* mRNA in the hippocampal neurons correlates with animal performance on hippocampus-associated cognitive tasks (Guzowski et al., 2001). In my current experiment, an increased brain expression of *Arc* and *Egr1* in prenatally Dex-exposed rats is potentially consistent with facilitation of the reversal learning and the DMTP task. Taken together, the current results highlighted a central role of synaptic function in prenatal glucocorticoid programming, suggesting that prenatal glucocorticoid exposure may influence the synaptic development of the offspring, leading to permanent alterations in synaptic strength and brain function, and thus inducing increased vulnerability to neuropsychiatric disorders in later life.

In an effort to elucidate the possible role of promoter DNA methylation in mediating the long-term effects of prenatal Dex exposure on brain *Arc* transcription, I examined DNA methylation across 4 loci of the *Arc* promoter and intragenic regions in the E20 and adult PFC samples using direct bisulfite Pyrosequencing.

The *Arc* gene CpG rich regions encompass both the promoter and the intragenic region. I examined 34 individual CpG sites that span the promoter region (detected by *Arc_P1* and *Arc_P2*) and the downstream CDS region (detected by *Arc_GB1* and *Arc_GB2*). Overall there was no effect of prenatal Dex treatment on DNA methylation at the examined CpG sites in both the E20 and adult PFC. The pattern of DNA methylation at these CpG sites was similar between the E20 and adult PFC. The average site-specific methylation levels at the promoter and intragenic regions ranged between 3% and 35%, which is compatible with a previous study (Penner et al., 2011). Thus, it seems that the *Arc* gene region may be constitutively methylated at low levels and DNA methylation may not correlate with gene expression. It is therefore likely that other epigenetic regulatory mechanisms such as histone modifications, instead of DNA methylation, may be responsible for determining the altered *Arc* expression in prenatally Dex-exposed rats.

In summary, my current experiment suggest that prenatal Dex exposure in rats induces long-term changes in brain gene expression involved in synaptic plasticity and memory formation, providing evidence that alterations in brain *GR/MR* expression and synaptic function may partly underlie the long-term alterations in brain function and behaviour induced by prenatal glucocorticoid treatment.

**Chapter 6 Mechanisms of programming: Insights
into retrotransposons and imprinted genes**

6.1 Introduction

Epigenetic dysregulation represents a plausible link between early life experience and long-lasting effects on cognition and mental health. As discussed in 1.4.2.1, there is evidence that the epigenetic signatures of the entire mammalian genome (epigenome) undergo precise, coordinated reprogramming at distinct stages of development. These developmental reprogramming events are characterized by remarkable changes of epigenetic marks, which may render these time points more susceptible to environmental perturbations compared to the fully differentiated state. Evidence of environmental influence on the epigenome during development is discussed in 1.4.3.

During developmental reprogramming, two epigenetic-related processes—retrotransposition and parental imprinting—undergo specific epigenetic control. They are exempted from the first wave of global DNA demethylation for the benefit of repression of transposon activities and maintaining parental imprinting (Seisenberger et al., 2013). Environmental perturbations such as Dex exposure during this critical time may have an influence on these two tightly regulated processes, and thus endanger genomic integrity and affect brain development and function.

Apart from developmental period, the activities of these two processes are still regulated and maintained mainly by epigenetic mechanisms and disrupted processes of each have been implicated in neuropsychiatric disorders (discussed below in 6.1.1 and 6.1.2).

6.1.1 Transposable elements (TEs)

Transposable elements (TEs) are mobile DNA pieces that can move and spread within (and sometimes between) genomes. They are classified by whether they mobilize via a DNA (DNA transposons) or an RNA (retrotransposons) intermediate. Retrotransposons are then further divided into long terminal repeat (LTR) and non-LTR retrotransposons depending on whether they contain or lack a LTR. DNA transposons move through a ‘cut-and-paste’ mechanism, whereas retrotransposons move through a ‘copy-and-paste’ mechanism via reverse transcribing and inserting into new genomic locations (Cordaux and Batzer, 2009).

Over the course of evolution most TEs have lost their ability to mobilise, however non-LTR retrotransposons, including the long interspersed element-1 (L1 or LINE-1) are the only TEs that are currently active in the human genome today (Cordaux and Batzer, 2009). As a result of their persistent activity and accumulation over tens of millions of years, these non-LTR retrotransposons have had a dramatic influence on the evolution of primate genomes (Cordaux and Batzer, 2009). TE mobilizations contribute to genomic innovations by generating genomic instability and rearrangements such as deletions, duplications and inversions (Levin and Moran, 2011). In addition, their insertions into the genome can influence genomic function by affecting the expression of nearby genes (Han et al., 2004). These retrotransposition events are important drivers for evolution (Kazazian, 2004), but at the same time pose threats to the genomic integrity and human health (Belancio et al., 2008).

Among them, L1s are the only autonomous TEs (able to mobilize on their own) in the human genome. There are about 500,000 copies of L1 in human genome, accounting for 17% of its DNA content (Lander et al., 2001). The canonical L1 element consists of two open reading frames (ORF1 and ORF2) flanked by a 5′ untranslated region (UTR) containing an internal RNA polymerase II (RNAPII) promoter and a 3′ UTR containing an oligo(dA)-rich tail (AAA)

preceded by a polyadenylation signal. ORF1 encodes a RNA-binding protein and ORF2 encodes a protein with endonuclease and reverse-transcriptase activities (Babushok and Kazazian, 2007). However of the 500,000 L1 copies most are not active due to truncations, internal rearrangements and mutations within the ORFs, and it has been estimated that only an average of 80-100 L1 copies are actually competent for retrotransposition in the human genome (Brouha et al., 2003). In addition, their retrotransposition activities are further regulated by various cellular processes including transcriptional and post-transcriptional silencing (Rodriguez and Burns, 2013).

Despite these defensive mechanisms, L1 retrotransposition events occur in the germ line and during embryogenesis (Kano et al., 2009), leading to heritable and non-heritable genetic diseases (Chen et al., 2005, Kazazian et al., 1988). The rate of this has been estimated as approximately 1 insertion for every 20 births in humans based on disease-causing *de novo* insertions (Kazazian, 1999). In addition, there is evidence that L1 retrotransposition activities take place in somatic tissues (Belancio et al., 2010), which are potentially mutagenic and have been implicated in the pathogenesis of cancer (Iskowitz et al., 2010, Rodriguez and Burns, 2013).

However, recent studies suggest high levels of L1 expression and retrotransposition take place in neuronal progenitor cells under physiological conditions in rodents and humans, which might contribute to neuronal differentiation and neuronal somatic mosaicism (Muotri et al., 2005). Indeed, increased copies of L1 were found in adult brain tissues compared to other peripheral tissues (Coulaf et al., 2009). In addition, L1 retrotransposition in the hippocampus has been implicated in experience-dependent neuronal plasticity in mice (Muotri et al., 2009). However, dysregulation of L1 activities, possible via epigenetic processes, has also been associated with neurodevelopmental diseases (Bundo et al., 2014, Muotri et al., 2010).

In summary, L1 expression and retrotransposition occur during embryogenesis and in the adult brain. Despite possible benefit for brain development, neuronal diversity and behaviour

(Singer et al., 2010), these retrotransposition events are potentially mutagenic. Dysregulated L1 activities, triggered or precipitated by prenatal events, may be involved in mediating the increased risk for later neuropsychiatric disorders (Thomas et al., 2012).

6.1.2 Imprinted genes

Imprinted genes are defined by their parent of origin specific monoallelic expression as a consequence of differential epigenetic markings of the paternal and maternal alleles established during parental gametogenesis (Reik and Walter, 2001). Many imprinted genes are highly expressed in the brain (Davies et al., 2005). Brain-expressed imprinted genes are crucial not only to early development, but also to brain function and behaviour (Kopsida et al., 2011).

Multiple lines of evidence suggest aberrant genomic imprinting play a role in neuropsychiatric disorders. The first evidence comes from studies of single-locus syndromes that have a well-understood molecular basis of disrupted genomic imprinting, including Prader-Willi syndrome, Angelman syndrome, Turner syndrome and Rett syndrome (Kopsida et al., 2011). These genetic syndromes encompass essential endophenotypes of neuropsychiatric disorders, including stereotyped behaviours, behavioural inflexibility, attentional deficits, cognitive deficits, psychosis, language and social impairments (Badcock and Crespi, 2006). The genomic basis to these individual endophenotypes is however not clear.

Evidence linking genomic imprinting to neuropsychiatric disorders also comes from the study of ASD. Firstly, autistic-like behaviour is commonly found in subjects with Angelman syndrome (AS) and Rett syndrome (Summers et al., 1995, Weaving et al., 2005). The incidence of AD is found much higher in individuals with these single-locus syndromes (Cohen et al., 2005). In addition, the chromosomal regions that linked to ASD on genome scans encompass those imprinted regions that have been implicated in AS (15q11-q13) (Cook et al., 1997) and Rett syndrome (7q22) (Cukier et al., 2009), suggesting common molecular lesion involving aberrant genomic imprinting may underlie these phenotypically related disorders (Kopsida et al., 2011).

Moreover, it has been suggested that genomic imprinting may contribute to the sex-bias in incidence rate and clinical presentations of related neuropsychiatric disorders. The idea is that males inherit a single X chromosome from their mother, thus that paternally expressed X-linked imprinted genes are never expressed in males. On the other hand, females inherit X chromosomes from both their parents and the expression of X-linked imprinted genes from paternal or maternal genomes depend on their X-inactivation status (Davies et al., 2006). Studies with Turner syndrome suggest that paternally and maternally X-linked imprinted genes have disparate effects on cognitive and behavioural phenotypes (Davies et al., 2006, Skuse et al., 1997), therefore they may contribute to the sex differences in vulnerability and presentation of neuropsychiatric disorders. Collectively, the above evidence supports the hypothesis that imprinted genes are implicated in the pathogenesis of neuropsychiatric disorders.

Imprinted genes usually cluster into large chromosomal domains, with maternally expressed genes alongside paternally expressed genes. Imprinting within these clusters is often regulated by differentially methylated regions (DMRs), especially the primary germ-line derived DMRs, in which the differential methylation is laid down in the germlines (Edwards and Ferguson-Smith, 2007). By contrast, secondary DMRs are those where differential methylation is established after fertilization. These secondary DMRs sometimes coordinate with the primary DMRs in the regulation of imprinted gene expression. For example, the imprinting control at the *Igf2/H19* region depends on long range chromatin interactions mediated by transcriptional repressors between the primary *H19*-DMR and the secondary *Igf2*-DMRs (Lopes et al., 2003). The *Dlk1-Dio3* imprinting region also contains a primary intergenic germline-derived DMR (IG-DMR) located 75 kb downstream of *Dlk1*, and a secondary *Gtl2*-DMR. Both DMRs are hypermethylated on the paternal allele and hypomethylated on the maternal allele in somatic cells. The IG-DMR is shown to function at the top of the hierarchy

and controls the methylation pattern of this imprinted gene cluster (Lin et al., 2003, Takada et al., 2002).

In the current project, I investigated the developmental and tissue-specific expression of several candidate imprinted genes in the E20 frontal lobe, adult PFC and adult hippocampus of the prenatal Dex exposure rat model. Loci of interest included: the *Dlk1-Gtl2* region located on human chromosome 14q32 (mouse distal 12), the Prader-Willi/Angelman (PWS/AS) region located on human chromosome 15q11-13 (mouse 7C), the *Grb10* region located on human chromosome 7p11.2-p13 (mouse 11) and the *Igf2/Cdkn1c* region located on human chromosome 11p15 (mouse 7). I further explored the possible effect of prenatal Dex treatment on DNA methylation at the *Igf2*-DMR2 and IG-DMR region using bisulfite Pyrosequencing.

Dlk1-Dio3 region

The *Dlk1-Dio3* locus harbours at least three paternally expressed protein-coding genes (*Dlk1*, *Rtl1*, and *Dio3*), and seven maternally expressed ncRNAs (*Meg3/Gtl2*, *Anti-Peg11*, *Meg8*, *Irm*/'*Rian*', *AK050713*, *AK053394* and *Meg9/Mirg*) (Hagan et al., 2009). I investigated the expression of three candidate genes within this cluster, including delta-like homolog 1 (*Dlk1*), iodothyronine deiodinase 3 (*Dio3*) and gene-trap locus 2 (*Gtl2* or *Meg3*).

DLK1 is a member of the Notch signalling pathway (da Rocha et al., 2008) and has been implicated in the differentiation of midbrain dopaminergic neurons (Christophersen et al., 2007). It is paternally expressed and its expression can be found in many adult mouse tissues, including brain, kidney, testis, and thymus (Hagan et al., 2009). *DIO3* encodes a protein that protects developing tissues from excessive amounts of thyroid hormone (Tsai et al., 2002). *Dio3* is preferentially expressed from the paternal allele in the mouse fetus (Hernandez et al., 2002) and it is less expressed in the adult brain than other tissues (Tierling et al., 2006). *MEG3*

(*Gtl2* in mouse) encodes a long noncoding RNA (lncRNA) that has a putative role of tumour suppressor (Zhou et al., 2012). *Gtl2* is maternally expressed and is highly expressed in the adult mouse brain (Tierling et al., 2006).

Prader-Willi /Angelman region (PWS/AS region)

Prader–Willi syndrome (PWS) and Angelman syndrome (AS) are caused by reciprocal deficiency of imprinted gene expression from the paternal and maternal alleles at human chromosome 15q11-q13. Clinically this locus is associated with a variety of behavioural and cognitive endophenotypes found in PWS/AS and related neuropsychiatric disorders, including aggression, behavioural inflexibility, attention deficits and psychosis (Soni et al., 2007).

The PWS/AS region harbours a cluster of imprinted genes, including the paternally expressed gene small nuclear ribonucleoprotein polypeptide N (*SNRPN*) and maternally expressed gene ubiquitin-protein ligase E3A (*UBE3A*). *SNRPN* is the most complex gene in the region, consists of 10 exons and serves as a host for a number of small nucleolar RNA (snoRNA) genes, including *SNORD116* (also known as *HBII/RBII/mbii-85*), which has been linked to key characteristics of the PWS phenotype (Buiting, 2010). On the other hand, *UBE3A* is the AS gene and is maternally expressed in the human brain. *UBE3A* has been implicated in ubiquitination of tumour suppressor protein p53 (Scheffner et al., 1993) and a number of other proteins involved in DNA repair and cell-cycle progression (Srivenugopal and Ali-Osman, 2002). In addition, *UBE3A* also function as a steroid receptor co-activator that might modulate the effects of sexual hormones on brain development and function (Wilkinson et al., 2007).

This region is conserved on mouse chromosome 7. Similar to humans, *Snrpn* is paternally expressed in the mouse brain and imprinted into adulthood (Runte et al., 2004). By contrast, the monoallelic expression of *Ube3a* in the mouse brain is confined to distinct areas such as

Purkinje neurons, the hippocampus and the mitral cells of the olfactory bulb. *Ube3a* exhibits biallelic expression in other parts of the brain and in other tissues (Albrecht et al., 1997).

Grb10 region

Growth factor receptor-binding protein 10 (*GRB10*) is mapped to human chromosome 7p11.2-p13 and has been suggested as a candidate gene for Silver-Russell syndrome (SRS), which is characterized by pre- and postnatal growth failure (Monk et al., 2000). *GRB10* encodes a SRC homology domain 2 (SH2)-containing adaptor protein, which can bind to several tyrosine kinases and growth receptors (He et al., 1998, Mano et al., 1998). It has been postulated that GRB10 is involved brain development by interacting with these receptors and downstream signalling molecules.

The mouse homologue (*Grb10/Meg1*) maps to the imprinted region of proximal mouse chromosome 11. It is preferentially expressed from the maternal allele in the periphery and loss of expression leads to significant fetal and placental overgrowth (Charalambous et al., 2003). By contrast, *Grb10* is paternally expressed in the mouse brain from fetal to adult life (Arnaud et al., 2003) and ablation of this imprinting in the brain leads to increased social dominance (Garfield et al., 2011). Thus, *Grb10* appears to influence distinct physiological processes, fetal growth and adult behaviour, by reciprocal expression from the two parental alleles in different tissues (Garfield et al., 2011, Monk et al., 2009).

Igf2 and Cdkn1c region

The human chromosome 11p15 harbours two clusters of imprinted genes involved in the regulation of prenatal growth. This imprinted region is conserved on mouse chromosome 7. In my study, I investigated the expression of insulin-like growth factor 2 (*Igf2*) and cyclin dependent kinase inhibitor 1C (*Cdkn1c*) from this imprinted region in my rat model.

Igf2 and *Cdkn1c* have antagonistic effects on growth, with the paternally expressed *Igf2* gene encoding an embryonic growth factor that promotes placental and embryonic growth (Randhawa and Cohen, 2005) and the maternally expressed *Cdkn1c* gene encoding a negative regulator of cell proliferation that inhibits growth (Andrews et al., 2007). In addition to its role in brain growth and development (Pidsley et al., 2010), there is evidence that *Igf2* is involved in important brain functions including memory consolidation and enhancement in rats (Chen et al., 2011). The imprinting status of *Igf2* is heterogeneous in the brain. In humans, *IGF2* exhibits biallelic expression in the fetal brain and a complex regional-specific parental allelic effect in the adult brain (Pham et al., 1998). In mouse, it has been reported that *Igf2* expression demonstrates a paternal bias in the fetal brain and a maternal bias in the adult PFC and hypothalamus regions (Gregg et al., 2010).

6.1.3 Hypotheses and aims

I hypothesise that fetal overexposure to glucocorticoids during pregnancy may modulate the epigenetic signatures of the brain, and the effect of which may be maintained through subsequent cell divisions and persist into adult life. In particular, I hypothesise that prenatal glucocorticoid overexposure may affect the epigenetic regulation of important processes during development, leading to sustained alterations in retrotransposon activities and imprinted gene expression in the brain, and therefore interfering with brain function and behaviour.

In this chapter I aimed to explore the possible effects of prenatal Dex treatment on candidate gene expression involved in retrotransposon activities and genomic imprinting. I also aimed to study the prenatal glucocorticoids effects on DNA methylation at regulatory regions of imprinted genes by direct bisulfite Pyrosequencing. Collectively, these experiments may provide insights into the underlying mechanisms of prenatal glucocorticoid programming.

6.2 Method

6.2.1 Quantitative real-time PCR

The same cDNA samples as described in 5.2.3 were used here for gene expression analysis. Briefly, 800ng of total RNA was obtained (n=CON:10, DEX:8 for E20 frontal lobe; n=CON:9, DEX:10 for adult PFC; n=CON:10, DEX:10 for adult hippocampus), DNase-treated and converted to cDNA. The qPCR was performed as described in 2.3.4.2, using self-designed UPL™ assays, PrimeTime® qPCR assays or Taqman® primer-probe mix. Data were normalized to *Hprt* for adult hippocampus samples, and to the mean of the three housekeeping genes (*Hprt*, *G6pd* and *Tbp*) for the E20 and adult PFC samples.

6.2.2 DNA methylation analysis

The same bisulfite-treated DNA samples as described in 5.2.5 were used here for DNA methylation analysis (n=CON:10, DEX:10 for E20 frontal lobe and n=CON:8, DEX:9 for adult PFC). 1µl of bisulfite converted DNA samples were amplified using primers that targeted either the *Igf2*-DMR2 or the IG-DMR region. Schematic diagram of the methylated regions for analysis is demonstrated in Figure 6.1. PCR conditions were optimized for each assay to ensure a clear band of product with sharp contrast to any primer-dimer band (Table 6.1 and Table 6.2). PCR program consisted of rapid heating to 95°C for 10 minutes followed by 45 cycles of 20 seconds of denaturation at 95°C, 20 seconds of annealing at 56°C and 20seconds of elongation at 72°C. The samples were then cooled down to 72°C for 7 minutes for completion. Methylation of DNA was measured by Pyrosequencing using the PyroMark Q24 system (Qiagen) as described in 2.3.5.3.

6.2.3 Statistical analysis

Data were analysed using GLMs (JMP statistical software, SAS Institute, Cary, NC, USA). All data were checked for homogeneity of variance and normality of distribution. All statistical models investigated the main effect of Group. Litter (nested within group) was fitted as random variable to account for the use of more than one animal per litter. Data were presented as mean value per group \pm S.E.M. Results were considered significant if $p < 0.05$.

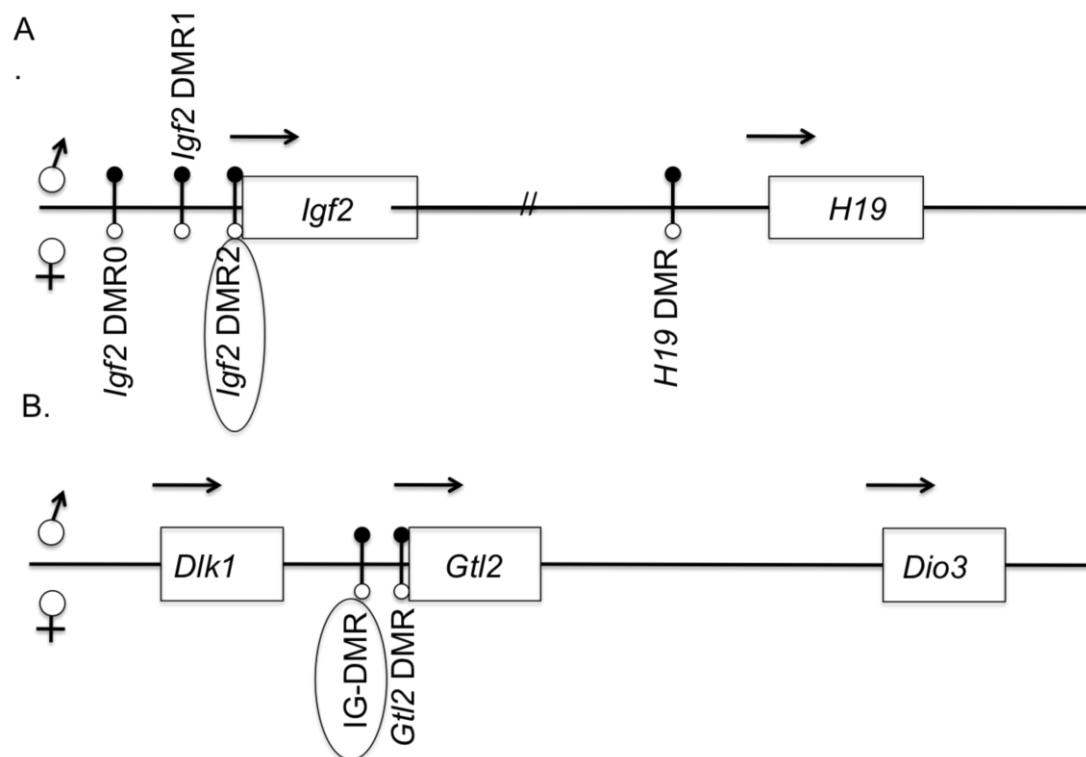


Figure 6.1 Schematic map of the *Igf2*/*H19* region and *Dlk1*/*Dio3* region

The arrow above a gene indicates the direction of transcription. The paternal allele is represented on the top and the maternal allele is on the bottom. DMRs are represented by small circles: a solid one indicates hypermethylation and an empty one indicates hypomethylation. In the current study, DNA methylation was assessed within circled regions *Igf2*-DMR2 and IG-DMR.

	IG-DMR1.2	IG-DMR1.3	IG-DMR2.3	IG-DMR2.4
	IG-DMR2.2			IG-DMR3.2
	IGF2 DMR2			
AmpliTaq MM	10µl	10µl	10µl	10µl
Primer mix (2µM)	3µl	2µl	5µl	4µl
DNA	1µl	1µl	1µl	1µl
Nuclease free water	6µl	8µl	4µl	5µl

Table 6.1 Optimised pyrosequencing PCR recipes for E20 frontal lobe samples

	IG-DMR1.2	IG-DMR1.3	IG-DMR2.3	IG-DMR2.4
	IG-DMR2.2			
	IG-DMR3.2			
	IGF2 DMR2			
AmpliTaq MM	10µl	10µl	10µl	10µl
Primer mix (2µM)	3µl	2µl	5µl	4µl
DNA	1µl	1µl	1µl	1µl
Nuclease free water	6µl	8µl	4µl	5µl

Table 6.2 Optimised pyrosequencing PCR recipes for adult PFC samples

6.3 Results

6.3.1 L1 expression in the brain

There was no effect of prenatal Dex treatment on L1 expression in the E20 frontal lobe. However, the level of L1 transcripts was significantly increased in the adult brain of prenatally Dex-exposed rats: there was an increased mRNA expression of L1 transcript ORF2 ($F_{1,13.06}=9.80$, $P=0.008$) and Rat1 ($F_{1,11.08}=4.85$, $P=0.050$) in the adult PFC, while there was increased expression of all three L1 transcript variants ORF2 ($F_{1,13.90}=7.36$, $P=0.017$), Rat1 ($F_{1,12.49}=13.20$, $P=0.003$) and Rn1 ($F_{1,14.79}=18.16$, $P=0.001$) in the adult hippocampus of prenatally Dex-exposed rats.

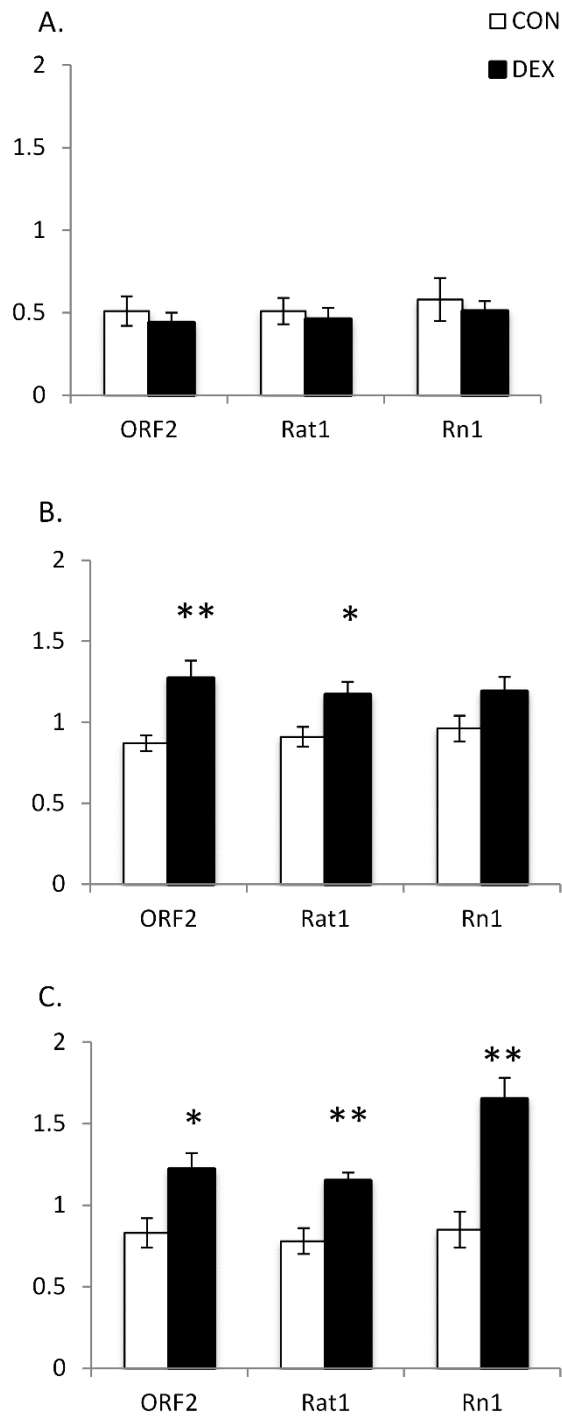


Figure 6.2 L1 expression in the brain

There was no difference between the two experimental groups in L1 expression in the E20 PFC (A); prenatal Dex exposure had an effect to increase mRNA expression of L1 transcripts in the adult PFC (B) and hippocampus (C). Data are presented as mean \pm S.E.M. $n=8-10/\text{group}$. * $P < 0.05$ versus control, ** $P < 0.01$ versus control.

6.3.2 Imprinted gene expression in the brain

There were no significant differences between the groups in brain expression of imprinted genes *Dlk1*, *Dio3*, *Gtl2*, *Snrpn*, *RBII85*, *Ube3a*, *Igf2* and *Cdkn1c* in the E20 frontal lobe, though there was a trend towards decreased expression of *Grb10* in the frontal lobe of Dex-treated embryos, ($F_{1,7.33}=5.22$, $P=0.055$). In the adult brain, prenatal Dex treatment had no obvious effect on imprinted gene expression in the PFC, however it had a broad impact on imprinted gene expression in the adult hippocampus. There was an increased hippocampal expression of *Dlk1* ($F_{1,14.35}=6.67$, $P=0.021$), *RBII85* ($F_{1,1.83}=45.76$, $P=0.027$), *Grb10* ($F_{1,14.73}=6.44$, $P=0.023$), *Cdkn1c* ($F_{1,14.99}=5.86$, $P=0.029$), and a trend towards increased expression of *Igf2* ($F_{1,15.00}=4.33$, $P=0.055$) in prenatally Dex-exposed rats.

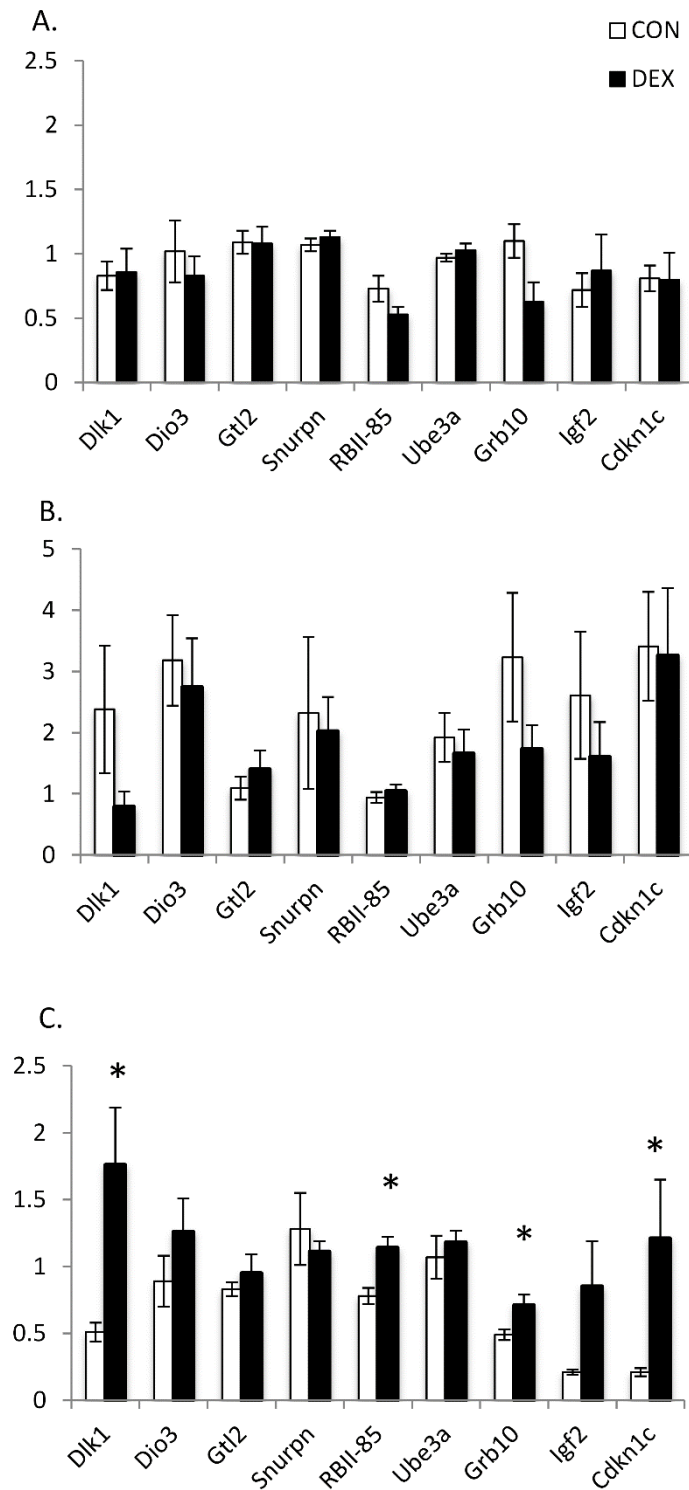


Figure 6.3 Imprinted gene expression in the brain

There was no effect of prenatal Dex exposure on brain imprinted gene expression in the E20 frontal lobe (A) and adult PFC (B); prenatal Dex exposure increased the expression of *Dlk1*, *Rbl1-85*, *Grb10* and *Cdkn1c* in the adult hippocampus (C). Data are presented as mean \pm S.E.M. n=8-10/group. * P < 0.05 versus control.

6.3.3 DNA methylation at the *Igf2*-DMR2 region

Figure 6.4 demonstrates the DNA methylation level at 9 individual CpG sites of the *Igf2*-DMR2 region. The efficiency of bisulfite conversion was calculated based on conversion rates of non-CpG cytosines and was around 90%.

There was no effect of prenatal Dex treatment on DNA methylation at the *Igf2*-DMR2 region in the E20 frontal lobe (A) and adult PFC (B). The level of DNA methylation averaged at $25.26 \pm 14.87\%$ for the E20 frontal lobe and $32.48 \pm 14.13\%$ for the adult PFC over the examined CpG sites.

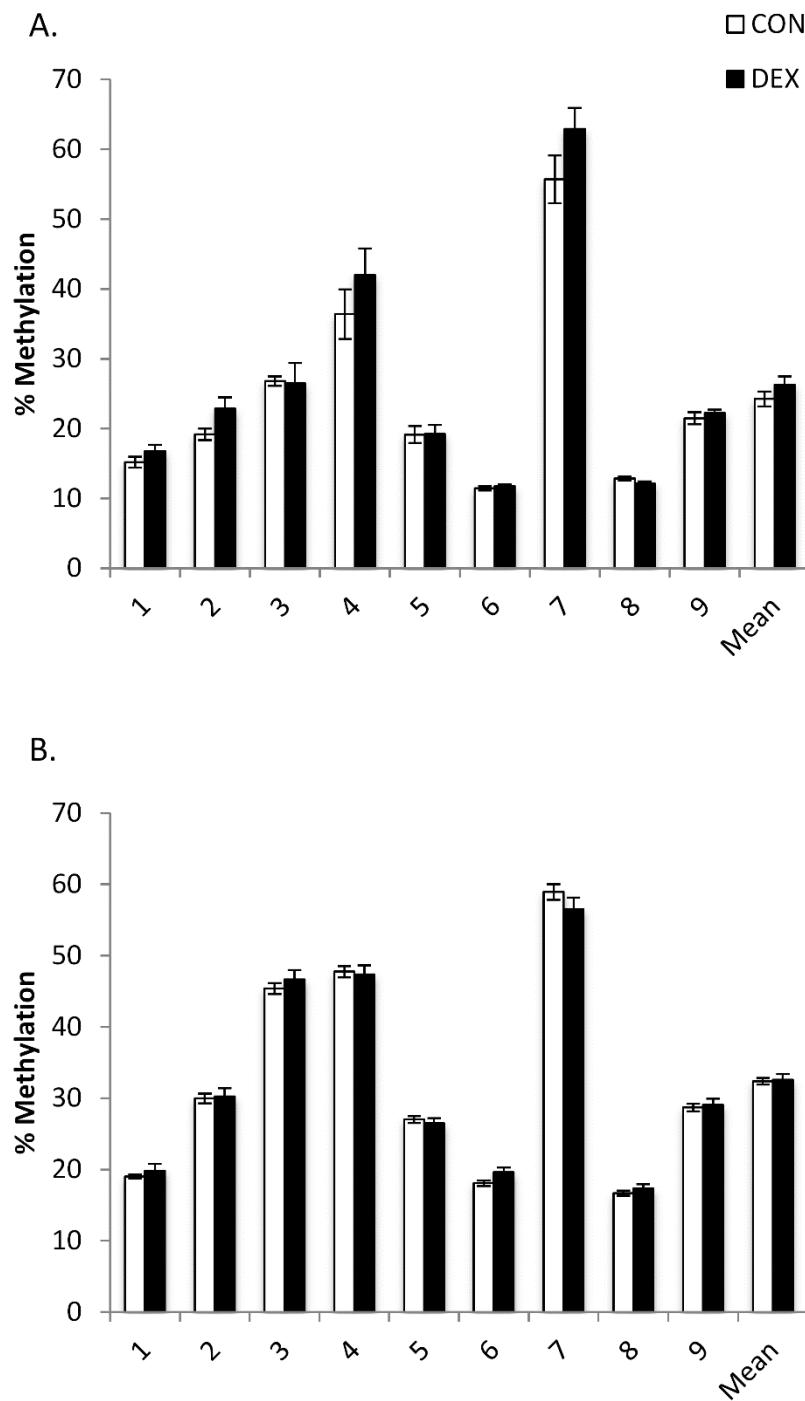


Figure 6.4 DNA methylation at the examined 9 CpG sites at *Igf2*-DMR2

There were no effects of prenatal Dex exposure on DNA methylation at *Igf2*-DMR2 in the E20 frontal lobe (A) and adult PFC (B). Data are presented as mean \pm S.E.M. n=8-10/group.

6.3.4 DNA methylation at the IG-DMR region

Figure 6.5 demonstrates the DNA methylation level at 6 CpG-rich loci at the IG-DMR region, which is the primary regulatory region for the imprinted *Dlk1-Dio3* locus. The efficiency of bisulfite conversion was calculated based on conversion rates of non-CpG cytosines and was around 90%.

There was no significant effect of prenatal Dex treatment on DNA methylation at IG-DMR in the E20 frontal lobe (A) and adult PFC (B). The average DNA methylation level at IG-DMR was $54.54 \pm 1.55\%$ for the E20 frontal lobe and $63.74 \pm 1.15\%$ for the adult PFC samples.

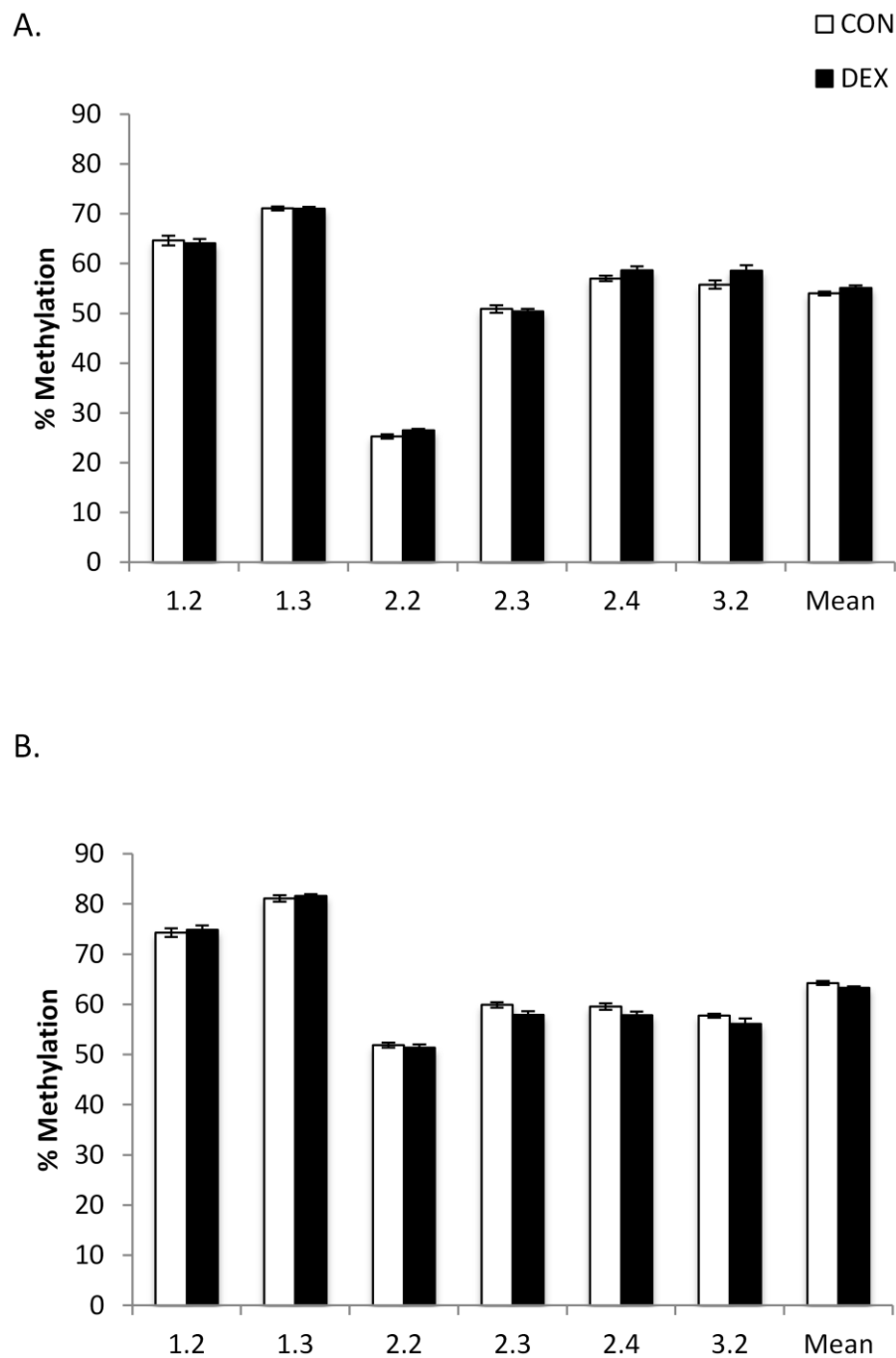


Figure 6.5 DNA methylation at the examined 6 CpG-rich loci at IG-DMR

There was no effect of prenatal Dex exposure on DNA methylation at IG-DMR in the E20 frontal lobe (A) and adult PFC (B). Data are presented as mean \pm S.E.M. n=8-10/group.

6.4 Discussion

In the present study, I investigated the effect of prenatal Dex administration on two epigenetic-related processes: retrotransposition and genomic imprinting. I examined the developmental stage- and regional-specific expression of L1 and candidate imprinted genes in dissected brain regions of the prenatal Dex exposure rat model. I also studied the DNA methylation at DMRs of the *Dlk1-Dio3* and *Igf2* imprinted regions. My results suggest that prenatal Dex exposure induced increased L1 transcription in the adult but not the E20 brain and had developmental stage- and regional-specific effects on brain imprinted gene expression in the male offspring.

My results revealed an increased expression of L1 in the adult brain of prenatally Dex-treated rats. There was unanimously increased expression of ORF2 but variable expression of the other two L1 transcript variants at different brain regions of prenatally Dex-exposed adult rat offspring. This is not surprising since ORF2 picks up any generic L1 copies including those truncated versions being transcribed from neighbouring gene promoters, whereas Rat1 and Rn1 target at the 5' UTR and only pick up copies that are being transcribed from their own promoters. The increased brain L1 transcription was only evident in adult, but not at an earlier developmental stage (E20), suggesting that prenatal Dex treatment may induce susceptibility for increased L1 transcription in later life.

My current results are relevant to several previous studies. For example, in a recent study researchers reported that prenatal treatment with poly I:C to pregnant dams induces increased L1 copies in mice offspring (Bundo et al., 2014). The same group of researchers also reported increased L1 ORF2 copy number in neurons from postmortem PFC of schizophrenia patients. Further evidence indicates brain-specific L1 insertions in these patients preferentially affect genes related to synaptic function and schizophrenia (Bundo et al., 2014). In addition, there is evidence that disrupted epigenetic regulatory processes induce an increased susceptibility for

L1 transcription and retrotransposition in rodents and cells derived from human patients with Rett syndrome (Muotri et al., 2010).

The implications of the above evidence are several fold: a) prenatal environmental factors induce long-term susceptibility for increased L1 transcription and retrotransposition; b) epigenetic processes are involved in regulating L1 activities; c) increased L1 activities are potentially implicated in the pathogenesis of schizophrenia. Together with my current results, the existing evidence supports the hypothesis that L1 retrotransposition activities may be implicated in prenatal programming of neuropsychiatric disorders.

My current results on imprinted gene expression suggest that prenatal Dex treatment in rats had distinct effects on brain imprinted gene expression during development and in different regions of adult brain. For example, there was a trend towards decreased *Grb10* expression in the E20 frontal lobe of Dex-treated fetuses, however in a previous study *Grb10* was up-regulated in Dex-exposed fetal liver using a same animal model (Drake et al., 2011). The distinct effect of Dex treatment on *Grb10* expression in the fetal brain and liver may reflect its reciprocal pattern of imprinting in the brain (paternally expressed) and peripheral tissues (maternally expressed) (described in 6.1.2). In addition, my current results provided further evidence for the regional-specific parental allelic effects of brain imprinted gene expression, such that there was an increased *Grb10* expression in the adult hippocampus but not PFC of prenatally Dex-exposed rats. Similarly, the expression of *Dlk1* was significantly increased in the adult hippocampus of prenatally Dex-treated rats, but was down-regulated in the adult PFC according to the next generation RNA-Seq data (described in 5.3.2). These results together demonstrate the complex spatiotemporal-specific effect of prenatal Dex treatment on brain imprinted gene expression.

Interestingly, my results revealed an increased expression of *RBII-85* in the adult hippocampus of prenatally Dex-exposed rats. As described in 6.1.2, *RBII-85* (*HBII-85* or *SNORD116* in human) is one of the snoRNAs located in the introns of a large ‘host gene’ *Snrpn* and generated during the pre-mRNA splicing process. The specific effect of prenatal Dex treatment on hippocampal *RBII-85* expression, but not on its host gene *Snrpn*, suggests the regulation of this gene may be important for prenatal Dex programming. Previous studies using single gene knock-out mice have associated *RBII-85* function with core features of PWS phenotype such as growth and obesity (Skryabin et al., 2007), however it may also contribute to other atypical behavioural and cognitive phenotypes of PWS (Relkovic and Isles, 2013), such as attention deficits (Woodcock et al., 2009), working memory and executive impairments (Jauregi et al., 2007). It is therefore possible that the aberrant hippocampal expression of *RBII-85* in prenatally Dex-exposed rats may underlie some of the cognitive phenotypes observed on my previous behavioural measures.

Imprinted genes play important roles in fetal growth and development. In a previous study that used a same rat model, researchers reported that prenatal Dex affects the expression of a number of growth-related imprinted genes in the placenta and fetal liver, with possible implications in fetal growth and subsequent birth weight (Drake et al., 2011). In my current study, there was no indication of prenatal Dex treatment effect on growth-related imprinted gene expression in the fetal brain, including *Igf2* and *Cdkn1c*. The tissue-specific effect of prenatal Dex treatment on growth-related imprinted gene expression may indicate a protective mechanism of the organism for brain development, however this argument would be stronger if I had obtained additional evidence on brain weight of these animals.

As described in 6.1.2, the somatic monoallelic expression of imprinted genes usually depends on the regulation of DMRs, whose differential methylation patterns are established during gametogenesis and after fertilization. Aberrant DNA methylation at these regulatory regions

is associated with a loss of imprinting and overexpression of related genes. In addition, there is evidence that prenatal environmental factors can affect the DNA methylation at DMRs (Tobi et al., 2012, Drake et al., 2011).

Unfortunately due to a limitation in DNA samples, I was not able to explore the potential role of DNA methylation at DMRs in mediating the altered hippocampal imprinted gene expression in my prenatal Dex exposure rat model. However, using my available brain DNA samples, I studied the DNA methylation level at *Igf2*-DMR2 and IG-DMR in the E20 frontal lobe and adult PFC in rats. As expected, there were no effects of prenatal Dex treatment on DNA methylation at both DMRs. The DNA methylation level averaged around 50% at IG-DMR in both E20 and adult PFC tissues, indicating that this region is differentially methylated in the frontal cortical region of the brain. Surprisingly, my results revealed that an average DNA methylation level around 30% at *Igf2*-DMR2 in the E20 and adult PFC, suggesting that this region is hypomethylated in the frontal cortical region of the brain. There was also a small increase in average DNA methylation level at both DMRs in the adult PFC compared to the E20 frontal lobe. While it may be true that these two DMRs become more methylated with aging, it could also be a reflection of different composition of brain cell types at different developmental stage, since different cell types may have different signatures at these DMRs.

In summary, my current results suggest that prenatal exposure to Dex induced increased L1 transcription in the adult brain. Prenatal Dex treatment also had spatiotemporal-specific effects on brain imprinted gene expression. Collectively, these results suggest that prenatal Dex exposure may induce long-term susceptibility to dysregulations in retrotransposon activities and imprinted gene expression in the brain, which may contribute to the development of neuropsychiatric disorders.

Chapter 7 Discussion

7.1 Summary

The current study aimed to obtain experimental evidence on the long-term safety of antenatal glucocorticoid treatment in the clinic, and to justify the validity of using the prenatal Dex exposure rat model for studying neuropsychiatric conditions in humans. In this thesis, I investigated the long-term effect of prenatal Dex exposure during the last week of gestation in rats on stress responses and cognition in adult male offspring. I also studied the possible molecular mechanisms associated with the behavioural phenotypes. Finally, I explored the possible role of two epigenetic-related processes in mediating the prenatal Dex programming effects in rat offspring.

The main findings of my current study can be summarized as below:

1. Dex exposure during the last week of gestation reduced birth weight in rat offspring. The reduced body weight persisted till adulthood in male offspring.
2. There was no effect of prenatal Dex exposure on a) basal and stress-induced plasma CORT levels and b) unconditioned fear and anxiety-related behaviour in the adult offspring.
3. Prenatal Dex exposure affected cognition in the adult male offspring, including a) contextual fear conditioning and b) within-task flexibility on water maze tasks. However, spatial reference memory, working memory and attentional set-shifting was not affected by prenatal Dex treatment.
4. The behavioural phenotypes were associated with altered expression of genes involved in synaptic plasticity and memory formation in the adult brain of prenatally Dex-exposed rats.
5. Prenatal exposure to Dex induced increased L1 transcription and altered imprinted gene expression profile in the adult brain.

7.2 Discussion

7.2.1 Behavioural results

In this thesis, I investigated the long-term neurobehavioral consequences and possible molecular implications of prenatal Dex exposure in adult male offspring in rats. My results suggest that prenatal Dex exposure had no effect on HPA axis and behavioural responses to an acute stress, it however affected hippocampus and PFC-associated cognition in the adult male offspring.

Despite previous reports that prenatal stress leads to stable changes in stress response systems and induces susceptibility to develop fear and anxiety-related behaviour after adult stress exposure, there is no evidence from my current study that prenatal Dex treatment affects the HPA axis and behavioural responsivities to stress in adult male offspring. Discrepancies in experimental findings may arise from variations in animal strain, prenatal stress regimen, age and sex of offspring and specific test conditions. It is interesting to note that despite using a same rat model my current elevated plus-maze and open field results contradicted with a previous study, where prenatally Dex-treated male rat offspring were found to have a trend towards decreased open arm entries on an elevated plus-maze and significantly decreased ambulation over a 12 min open field test (Welberg et al., 2001). In my current study there were no significant findings from the elevated plus-maze and possible contributing factors have been discussed in 3.4. In the open field test, my current data show a tendency towards increased ambulation in prenatally Dex-exposed rats, indicating potential dexamethasone programming effects on spontaneous locomotion in adult offspring, in line with most previous studies (Diaz et al., 1997, Wilson et al., 2013). The contradictory findings in an open field test between my current study and the Welberg study could be due to differences in aversiveness of the task such as illumination on the apparatus, and more importantly due to the protocol such as the period of time over which ambulation was examined (5 min in my experiment and 12 min in

the Welberg study). For example, there is evidence from previous study that prenatally stressed male rats exhibit decreased overall exploratory activity despite an higher initial activity in an open field (Poltyrev et al., 1996a). Overall, my current study does not support the hypothesis that prenatally Dex-exposed individuals are at increased risk of developing stress-related psychopathological changes in adulthood.

My current results also suggest that prenatal Dex exposure in rats impairs the development of context-dependent fear conditioning in adult male offspring. Contextual fear conditioning depends on the neural circuit between the hippocampus and the amygdala (Rudy et al., 2004). There is evidence that hippocampal synapse activity is implicated in fear conditioning (Rao-Ruiz et al., 2015). Thus, the impaired contextual fear conditioning in prenatally Dex-exposed animals could indicate potential deficits in hippocampal synapse function.

In this thesis, cognitive flexibility is assessed by a) fear extinction in a contextual fear conditioning test; b) reversal learning and DMTP in a Morris water maze; and c) attentional set-shifting in an ID/ED test. The current data for fear extinction is inconclusive and future experiments are required to elucidate this further. The current results suggest that prenatally Dex-treated rats are faster in responding to changes in stimulus-reward associations in water maze tasks. Similar behavioural effects following prenatal Dex exposure have been reported in Marmosets (*Callithrix jacchus*, primates), in which late gestational Dex treatment induced enhanced reversal learning of stimulus-reward contingencies in adolescent monkeys (Hauser et al., 2008). In addition, the current results suggest prenatal Dex treatment facilitates behavioural flexibility on water maze tasks without affecting attentional shifts. Lesion studies in monkeys and rats suggest that different regions of the PFC mediate different aspects of cognitive flexibility, for example there is evidence lesions to the rat orbital prefrontal cortex produce deficits in reversal learning in the absence of deficits in shifting attentional sets (McAlonan and Brown, 2003, Dias et al., 1996). Thus, my current results may indicate

dissociable effects of prenatal Dex treatment on cognitive processing involved in different aspects of behavioural flexibility.

As discussed in 4.4, the current results suggest that increased behavioural flexibility in prenatally Dex-exposed rats are not associated with memory deficits; instead it indicates deficits involving non-mnemonic processes such as attention and inhibitory control. Similar abnormalities are characterized in patients with psychiatric disorders such as ADHD (Barkley, 1997), including impaired sustained attention (Berwid et al., 2005) and endogenous response control (van den Bergh et al., 2006). Interestingly, in one human study researchers investigated the cognitive consequences of maternal anxiety during pregnancy on adolescent performance on a matching-to-sample task. The adolescent offspring of mothers who had higher levels of gestational anxiety responded significantly faster, but with more errors, compared to the adolescent offspring of mothers who had lower levels of anxiety during pregnancy. Working memory, inhibition of a prepotent response (perseveration tendencies) and visual orienting of attention were not impaired between the two groups (Van den Bergh et al., 2005, Mennes et al., 2006). These cognitive outcomes are very similar to what I found in the current study, including no effect on working memory and attentional set-shifting and faster responding to changes in task-requirements in prenatally exposed individuals. The authors of the above-mentioned human study suggest that the behavioural phenotype in adolescents exposed to higher antenatal maternal anxiety is indicative of impaired endogenous inhibitory control (Van den Bergh et al., 2005). In a follow up study, the same group of researchers suggest a link between antenatal maternal anxiety and impairments in sustained attention or self-regulation in adolescents (van den Bergh et al., 2006). It is however important to note that cognitive processes differ significantly between humans and animals, such that these results are not directly comparable. Overall although the prenatal Dex exposure rat model share some phenomenological similarities to related neuropsychiatric disorders, most of the results of the current set of experiments are not what we would have expected, and the validity of using this

animal model for studying risks and mechanisms of related human conditions needs to be further justified.

7.2.2 Molecular results

In this thesis, I also explored possible molecular mechanisms underlying the cognitive behavioural phenotypes induced by prenatal Dex treatment. As discussed in 5.1, brain GR and MR expression mediate complimentary but also in part overlapping actions in stress responses, memory and behavioural functions (Korte, 2001, de Kloet et al., 1999). In my current study, prenatally Dex-treated rats exhibited increased *GR* expression in the hippocampus and increased *MR* expression in the PFC. Behaviourally, offspring of stressed dams exhibited normal stress hormonal response, impaired fear conditioning and increased behavioural flexibility in the Morris water maze tests. It is hard to combine all the evidence and give a consistent explanation in terms of brain *GR/MR* expression and the associated behavioural findings. Possible confounding factors may include discrete function of GR/MR expression at specific regions of the brain, for example there is evidence that forebrain *GR* knockout is associated with impaired HPA axis feedback and increased anxiety-like behaviour, whereas amygdala-specific *GR* disruption is associated with deficient fear conditioning (Arnett et al., 2011). In addition, any functional relevance of mRNA expression still depends on the actual level of protein translation and other molecular mechanisms may also take part in contributing to the behavioural phenotypes.

My current study also highlighted a role of synaptic function in mediating prenatal glucocorticoid effects. Previous studies suggest that hippocampal synaptic plasticity is implicated in contextual fear conditioning (Rao-Ruiz et al., 2015) and synaptic plasticity within the hippocampus-to-PFC connection is associated with PFC-dependent task performance (Cerqueira et al., 2007). In fact, there is evidence that prenatal stress impairs

hippocampal synaptic function in the adult offspring, associated with spatial learning deficits in the Morris water maze (Yang et al., 2006, Son et al., 2006). In my study, there was an increased baseline expression of *Egr1* and *Arc* in the adult brain of prenatally Dex-treated rats, together with impaired fear-related memory retention and enhanced behavioural learning and flexibility in Morris water maze tasks. Although there is evidence that resting levels of *Arc* transcription are associated with memory consolidation and retrieval (Penner et al., 2011), it is the activity-dependent IEGs induction that produces stable changes in synaptic strength, which is associated with behavioural learning and flexibility. Within the current data sheet it is hard to predict how an increased resting levels of IEGs expression might affect synaptic plasticity and contribute to the behavioural phenotypes during training.

In this thesis I also found altered imprinted gene expression profile in the adult hippocampus of prenatally Dex-exposed rats. However too little is still known about the function of imprinted genes in the adult brain, and brain-expressed imprinted genes seem to have disparate roles in the brain in addition to their common roles in the peripheral system (Garfield et al., 2011). It is therefore not yet possible to draw any conclusion on the behavioural implications of altered imprinted gene expression in the brain. Interestingly however, my results revealed increased L1 transcription in the adult brain of prenatally Dex-exposed animals. L1 expression was not increased in the E20 frontal lobe of the Dex-treated embryos, suggesting that prenatal Dex exposure induces susceptibility to increased L1 transcription in adults. Increased retrotransposon activities may endanger the integrity of the genome, and have been implicated in the pathogenesis of schizophrenia (Bundo et al., 2014).

Overall my current molecular results provide evidence that prenatal Dex exposure may interfere with synaptic development and retrotransposon activities in the offspring, leading to permanent alterations in synaptic strength and brain function, and thus inducing increased risk for neuropsychiatric disorders in later life. Further investigations into these molecular

processes may provide insights into the mechanisms through which prenatal environment evokes an impact on future mental health.

7.2.3 Prenatal programming and adaptations

Finally, the results of my current study again bring into focus the controversy of adaptive and maladaptive effect of prenatal programming. The current data suggest that prenatal Dex exposure induces impaired fear conditioning in the offspring. For animals living in the wild a failure to consolidate the fear memories of a predator's appearance, smell and location is apparently a disadvantage, which could result in a failure to predict and prevent future encounters with the predator, and thereby reduce the likelihood of survival. In addition, prenatally Dex-exposed rats show faster responding to changes in task-requirements on water maze tasks. Although this phenotype may associate with deficits in attention and inhibitory control, it is potentially an advantage to survive in a hazardous and ever-changing environment. This evidence lends support to the match-mismatch hypothesis, such that an individual who has been exposed to an aversive *in utero* environment is programmed to 'expect' an unfavourable environment after birth; if the later environment turns out to be challenging, the programmed traits can thus become a privilege (Nederhof and Schmidt, 2012). Overall my current results suggest that the outcome of early life programming is not always deleterious, and the pros and cons of prenatal programming are better to be evaluated in the context of both the individual and the current environment.

7.3 Future work

7.3.1 Behavioural experiments

In this thesis, I found no evidence of prenatal Dex treatment effects on basal and stress-induced plasma CORT levels 15 min after exposure to an acute stress. Previous studies suggest that prenatal stress may not affect basal or peak CORT levels after a stress exposure; it however induces prolonged CORT secretion and the elevated CORT levels 2 hr after stress exposure (Maccari et al., 2003, Henry et al., 1994, Vallee et al., 1997, Koenig et al., 2005). There is also evidence that prenatal stress affects hormone adaptation after repeated exposure to stress (Fride et al., 1986). It would thus be interesting to study the potential effect of prenatal Dex exposure on hormone adaptations such as the time-course of stress-induced hormone secretion after an acute and repeated stress. In addition, it might be interesting to look at other biochemical indicators of HPA axis function. For example, there is evidence prenatal stress leads to increased secretion of overall HPA axis hormones including circulating adrenocorticotrophic hormone (ACTH) and CORT (Takahashi and Kalin, 1991).

In terms of fear and anxiety-related behaviour, I have shown that prenatal Dex exposure had no effect on unconditioned fear and anxiety-related behaviour; however it affected fear-related memory consolidation in the adult male offspring. In future it would be interesting to explore other aspects of fear and anxiety-related behaviour, for example, fear potentiation and fear extinction. Fear potentiation is interesting because a) there is evidence that prenatal stress leads to decreased innate fear (Wilson et al., 2013), which may affect an animal's emotional evaluation of a stressful situation and may interfere with measures that depend on innate fear responses and b) there is evidence that prenatal stress induces behavioural differences only on a fear-potentiated, but not unconditioned elevated plus-maze and open field in rats (Estanislau and Morato, 2005, Poltyrev et al., 1996b). In terms of fear extinction, there is evidence that variable prenatal stress and restraint stress to pregnant dams induces impaired fear extinction

in adult rat offspring (Green et al., 2011b, Markham et al., 2010). It is therefore possible that prenatal Dex administration to pregnant dams might also have an effect on behavioural adaptation of conditioned fear in adult offspring.

In terms of the current water maze and ID/ED results, prenatal Dex treatment had no effect on attentional set-shifting, it however facilitated behavioural flexibility on water maze tasks involving non-mnemonic processes. The brain regions that have been mapped to the cognitive phenotype mentioned above include cortical and sub-cortical regions linked to the orbitofrontal cortex (Mennes et al., 2006, McAlonan and Brown, 2003). It would be interesting in future studies to explore prenatal Dex treatment effects on other cognitive aspects related to orbitofrontal cortex function. In addition, future experiments would enable us to dissociate potential contributions of attention and inhibitory control to the increased flexibility on water maze tasks. Possible experiments include a radial arm maze (Bimonte and Denenberg, 2000), a 5-choice serial reaction time task (Asinof and Paine, 2014), a two-choice operant task (Adriani et al., 2010) and a delay-discounting task (Evenden and Ryan, 1996).

Finally, it may be worth to consider possible postnatal maternal behaviour influence on prenatal programming. Some previous studies suggest that the prenatal programming effects are independent of postnatal maternal behaviour (Nyirenda et al., 2001, Holloway et al., 2013). By contrast, there is evidence that cross fostering by a control mother can reverse the effect of prenatal treatment on HPA axis activity and spatial learning (Maccari et al., 1995, Brabham et al., 2000), whereas cross fostering by a stressed mother can exacerbate the prenatal effects on offspring (Francis et al., 1999, Kuo et al., 2014). A cross-fostering paradigm in future studies would enable dissection of possible contributions of prenatal and postnatal maternal factors to programming.

7.3.2 Molecular experiments

In this thesis, I have shown that prenatal Dex exposure induced altered basal-state expression of candidate genes implicated in synaptic plasticity in the brain. There is evidence that synaptic plasticity is implicated in prenatal stress induced learning deficits (Yang et al., 2006) as well as human neuropsychiatric disorders such as schizophrenia (Hall et al., 2015). Given the role of genes such as *Arc* in plasticity and learning this would be a particularly interesting future avenue of investigation.

It has been reported that *Arc* mRNA is accumulated at the dendrites of stimulated neurons (Lyford et al., 1995). Its transcription is induced by neural activity specifically associated with active information processing, such that cellular imaging of *Arc* induction has become a robust method for detecting neural networks engaged by discrete behaviours (Guzowski et al., 2005). There is also evidence that the level of *Arc* transcription induced by behaviour training is associated with animal performance and behavioural flexibility (Guzowski et al., 2001). In response to neural activity, *Arc* mRNA is transported to dendrites and becomes enriched at the site of local synaptic activity, where ARC protein is locally synthesized. Translation of ARC protein at active synapses is considered the molecular basis for stabilising activity-dependent changes in synaptic efficacy that underlies learning and memory formation (Robertson, 1992). There is evidence that suppression of ARC protein translation impairs long-term memory consolidation without affecting task acquisition and short-term memory (Guzowski, 2002). On the other hand, over-accumulation of ARC leads to concomitant decrease in the number of glutamate receptors at excitatory synapses, which impairs synapse function and contributes to the cognitive impairments in human patients (Greer et al., 2010).

My current study shows an increased *Arc* mRNA in the adult brain of prenatally Dex-treated rats at resting conditions. In future it would be of interest to determine whether prenatal Dex

exposure alters the activity-dependent *Arc* induction after behaviour training. In fact, to fully character the prenatal Dex effects on *Arc* regulation and synaptic function, it is essential to decide the precise kinetics of *Arc* mRNA transcription and local protein translation at distal synapses, as well as *Arc* mRNA intracellular trafficking within the neuronal processes, ideally calls for fluorescence *in situ* hybridisation combined with quantitative methods such as RT-PCR in future studies.

In addition, my current study revealed increased L1 transcription in the adult brain of prenatally Dex-exposed rats. It is possible that increased L1 transcription might associate with increased retrotransposition events, which can be confirmed by increased L1 copy numbers in the genomic DNA. It would in addition be of interest to see if alterations in L1 activities are regulated by epigenetic mechanisms such as DNA methylation. Essays have been developed for measuring global L1 methylation levels using MethyLight (Weisenberger et al., 2005). It would also be of interest to investigate L1 activities and methylation profiles in the peripheral tissues and blood cells as potential biomarkers for neuropsychiatric disorders.

Finally, I have discussed in this thesis that stable epigenetic marks acquired during developmental time may serve as archives of early exposures with potential to improve our knowledge of the mechanisms of fetal origins of adult disease. Although my current study didn't provide direct evidence that epigenetics is involved in mediating the long-term effects of prenatal Dex exposure, future studies on epigenetic patterns of the brain after prenatal exposure to stress have a potential for identifying how environmental experiences or challenges evoke life-long impact on behaviour, brain function and disease susceptibility.

In summary, my current study provides evidence that prenatal exposure to stress hormones has complex effects on later behaviour and brain gene expression. My study also suggests a

role of synaptic function and retrotransposon activities in mediating the long-term impact of prenatal glucocorticoid exposure on mental health. Thus, prenatal exposure to glucocorticoids may have programming effect on synaptic development and retrotransposon activities that permanently affect brain function and behaviour, and thus leading to increased susceptibility to a range of neuropsychiatric phenotypes in later life.

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Appendix

General chemicals/solutions/kits

Sigma-Aldrich Ltd., Dorset, UK

Water (Molecular Biology Reagent)

Sodium acetate

Hydrogen peroxide solution

Ethylenediaminetetraacetic acid disodium salt
dihydrate

Diethyl pyrocarbonate

2-Mercaptoethanol

Antifoam A Concentrate

Guanidine thiocyanate

N-Lauroylsarcosine sodium salt

Trizma[®] base

Phenol solution pH 4.3 ± 0.2

Chloroform-isoamyl alcohol mixture

VWR International, Leicestershire, UK

Ethanol

Isopropanol

Bioline, London, UK

Agarose powder

Biotium Inc., California, USA

Gel-Red[™] nucleic acid stain

Promega Ltd., Southampton, UK

RQ1 RNase-Free DNase

RQ1 DNase 10× Reaction Buffer

RQ1 DNase Stop Solution

Qiagen, West Sussex, UK

RNeasy[®] Mini Kit

RNeasy[®] Plus Mini Kit

DNeasy[®] Blood & Tissue Kit

	Multiplex PCR Kit
	PyroMark Q24 Gold Reagents (5 x 24)
	PyroMark Annealing Buffer
	PyroMark Binding Buffer
	PyroMark Wash Buffer
	PyroMark Denaturation Solution
Invitrogen, Glasgow, UK	Customer Primers
	Qubit® RNA HS Assay Kits
	Qubit® dsDNA HS Assay Kits
	PureLink™ RNase A
	100bp DNA ladder
Applied Biosystems, Warrington, UK	TaqMan™ Gene expression assay
	AmpliTaq® Gold PCR Master Mix
	High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor
GE Healthcare Life Sciences, Sweden	Streptavidin Sepharose™ High Performance
Roche Diagnostics Ltd., West Sussex, UK	LightCycler 480 Probes Master
	Universal Probe Library Probes
Zymo Research, Irvine, USA	EZ DNA Methylation™ Kit
Agilent Technologies, Cheshire, UK	Agilent RNA 6000 Nano Kit

Equipment

Manufacturer	Equipment
Agilent Technologies, Cheshire, UK	Agilent 2100 Bioanalyzer system
Bio-Rad Laboratories Ltd., Hertfordshire, UK	Power Pack 200 agarose gel system
EMD Millipore	Milli-Q integral water purification system
Eppendorf, Cambridge, UK	5424 Bench centrifuge 5415R Bench centrifuge Grant bio LMC-3000 laboratory centrifuge
G-Storm Ltd., Somerset, UK	GS-1 Thermo Cycler
Invitrogen, Paisley, UK	Qubit [®] 2.0 Fluorometer
PRO Scientific, Connecticut, USA	PRO200 Homogeniser
Qiagen, West Sussex, UK	PyroMark Q24 system
Roche Diagnostics Ltd., West Sussex, UK	Roche [®] 480 Light Cycler
UVItec, Cambridge, UK	UviPro system

Software

Equipment

Agilent 2100 Bioanalyzer

Fear conditioning

LightCycler 480

Morris water maze

PyroMark Q24

Statistics

Software

2100 Expert Software (Agilent Technologies)

FreezeFrame software (Coulbourn Instruments, USA)

LightCycler 480 release 1.5 O SP3 (Roche)

Water maze software (Tracker- P. Spooner)

PyroMark Assay Design 2.0 software (Qiagen)

PyroMark Q24 analysis software (Qiagen)

JMP statistical software (SAS Institute, USA)

